CHARACTERIZATION OF A TOPOISOMERASE FROM *Ustilago maydis*

BY

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1982

To my father

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ACKNOWLEDGMENTS

I would like to extend my sincerest thanks to Dr. William Holloman for his guidance and genuine friendship. He will always be an inspiration to my work. In addition I would like to thank Drs. Kenneth Berns, Bert Flanegan, George Gifford, Nicholas Muzyzcka, and Gary Stein for their helpful suggestions and encouragement during the course of these studies.

Special thanks also go to the crew in D4-42. In particular, I would like to thank Drs. James Rusche and Michael Brougham for their support, friendship and great discussions (scientific and otherwise).

Finally, I would like to warmly thank the remaining students, faculty and secretarial staff in the Immunology and Medical Microbiology Department for making my stay here such an enjoyable one.

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KEY TO ABBREVIATIONS

ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
CTP	cytosine-5'-triphosphate
DBP	DNA binding protein
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
GTP	guanosine-5'-triphosphate
PMSF	phenylmethylsulfonylfluoride
poly(dA)	polydeoxyadenylic acid
poly(dC)	polydeoxycytidylic acid
poly(dG)	polydeoxyguanylic acid
poly(dT)	polydeoxythymidylic acid
RF I	form I, superhelical DNA
RF II	form II, nicked circular DNA
RF III	form III, linear DNA
RF IV	form IV, relaxed closed circular DNA
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	standard saline citrate (0.15 M NaCl, 15 mM sodium citrate)

Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF A TOPOISOMERASE FROM *Ustilago maydis*

By

Thomas Cardon Rowe

December 1982

Chairman: William K. Holloman
Major Department: Medical Sciences (Immunology and Medical Microbiology)

A topoisomerase from the lower eukaryote *Ustilago maydis* was isolated and characterized. By gel filtration, the topoisomerase has a native molecular weight of 270,000. The enzyme can remove both positive and negative superhelical turns from DNA in an ATP-independent reaction. Activity is stimulated by 10 mM Mg^{2+}, but is totally inhibited by Zn^{2+} at concentrations as low as 20 μM. The topoisomerase is active over a broad pH range from 5.5 - 8.0. The optimal temperature for activity is 20° - 30°C.

ATP inhibits topoisomerase activity and may reflect a means by which this enzyme can be regulated. Inhibition does not appear to require hydrolysis of ATP since several structural analogs of ATP also inhibit activity.

Histone H1 from calf thymus stimulated the topoisomerase greater than 25-fold. An H1-like protein has been isolated from *Ustilago* which also markedly stimulates the topoisomerase. This interaction may be important in regulating topoisomerase in cellular processes.

The topoisomerase breaks single-stranded DNA and forms a covalent complex with the 3' end of the DNA break. The covalent linkage appears to involve a phosphotyrosine bond.

The rec 2 mutant of Ustilago has decreased levels of topoisomerase activity possibly implicating this enzyme in eukaryotic genetic recombination.

CHAPTER I
INTRODUCTION

Introduction

Topoisomerases are enzymes which catalyze the interconversion of topological isomers of DNA by a concerted breakage and reunion of the DNA phosphodiester bonds (1,2). By altering the topological structure of DNA, topoisomerases are believed to play a key role in regulating the biological activity of DNA (3).

Although topoisomerases were initially identified by their ability to change the superhelical density of DNA, they have since demonstrated an ability to carry out other enzymatic gymnastics leading to an interconversion between knotted and unknotted forms of single- and double-stranded DNA as well as the catenation and decatenation of duplex DNA rings (1-6). Because of their adeptness at solving toplogical puzzles, topoisomerases are thought to play an important role in DNA replication, transcription and genetic recombination (1,3,5,7).

Mechanism of Topoisomerase Action

Topoisomerases derive their name from an ability to change the topological linking number, L, of covalently closed circular duplex DNA. The linking number is defined as the number of times one strand of the DNA duplex winds about the other strand when the DNA is forced to lie in a plane (7) and can be related to the number of Watson-Crick turns, β, and the number of superhelical turns, τ, by the following simple equation $L = \beta + \tau$ (8). The linking number for a closed circular

molecule remains constant unless the DNA backbone becomes broken. There are two basic ways topoisomerases can alter the topological linking number of DNA and they are designated as being type I or type II enzymes depending upon which mechanism they employ (1-3,5).

Type I Topoisomerases

A type I enzyme alters the topological linking number of steps of one by introducing transient single-strand breaks into the DNA resulting in a covalently linked enzyme-DNA intermediate involving a phosphotyrosine bond (9,10). Resealing of the break requires no energy cofactor but instead is thought to be driven by the energy stored in the covalent enzyme-DNA bond (11). With the exception of the lambda integrase topoisomerase, all prokaryotic type I enzymes tested form a covalent complex with the 5' end of the DNA (12). This is in contrast to eukaryotic type I topoisomerases and the lambda integrase topoisomerase which form a covalent complex with the 3' end of the DNA (13,14). Prokaryotic and eukaryotic enzymes also differ in their ability to relax positively and negatively supercoiled DNA. Although eukaryotic topoisomerases relax both positively and negatively supercoiled DNA (15,16), prokaryotic type I enzymes, excluding the lambda integrase topoisomerase (17), can remove only negative superhelical turns from DNA (11,18,19). Both eukaryotic and prokaryotic enzymes are able to catenate duplex rings of DNA if one of the rings of DNA involved in the reaction contains a preexisting nick (20,21). The prokaryotic topoisomerase from *E. coli* called *omega* protein also displays an ability to knot and unknot both single-stranded and duplex DNA circles (21). However, like the catenation reaction, the

introduction and removal of knots from duplex DNA circles requires that the DNA has a preexisting nick in one of the strands (21). Another reaction which is catalyzed by both eukaryotic and prokaryotic type I topoisomerases is the intertwining of complementary single-stranded rings of DNA to form covalently closed circular duplex DNA (22,23).

Type II Topoisomerases

Type II topoisomerases introduce transient double-strand breaks into DNA. Passing a strand through the break type II topoisomerases can change the topological linking number in steps of two, a process which is called sign inversion (24). Similar to type I enzymes, type II topoisomerases form a covalent enzyme-DNA complex involving a phosphotyrosine bond (9). In both eukaryotes and prokaryotes this linkage is with the 5' end of the DNA (9). The first enzyme discovered in this group, DNA gyrase, has the unique ability to catalyze the conversion of relaxed closed circular duplex DNA to a negatively supercoiled form, a process which requires hydrolysis of ATP (25). Gyrase is inhibited by the antibiotics nalidixic acid and novobiocin. The sensitivity of gyrase to these two antibiotics has been effectively used to establish the biological role of this topoisomerase in DNA replication, transcription and recombination (3).

Type II topoisomerases have been isolated from a variety of prokaryotic and eukaryotic organisms (26-28). They all share an ability to remove positive as well as negative supercoils from DNA (27,3). In addition, these enzymes can introduce and remove knots from double-stranded DNA as well as catenate and decatenate duplex DNA rings

(27,28). All of these reactions proceed in the absence of preexisting nicks in the DNA which is consistent with an enzyme mechanism involving double-stranded breaks in the DNA (24,27). Although all of the topological reactions catalyzed by eukarotyic and T4 type II topoisomerases require ATP, there are examples of type II activities which can occur in the absence of ATP, such as the relaxation of negatively supercoiled DNA by gyrase (29).

Role of Topoisomerases in Genetic Recombination

Having discussed some of the salient features of topoisomerases, how might those enzymes be involved in DNA recombination? One possible way topoisomerases might influence genetic recombination is by altering the superhelix density of DNA. Interestingly, it was studies on the role of superhelicity in lambda site-specific recombination that led to the discovery of DNA gyrase (26). Mizuuchi et al. found that superhelical lambda DNA served as a good *in vitro* substrate for recombination when it was incubated with *E. coli* extracts containing the lambda integrase protein (30). However, no recombination took place if superhelical lambda DNA was replaced by relaxed, nonsupercoiled lambda DNA, unless ATP was included in the reaction mixture. This was somewhat puzzling until it was discovered that ATP was required by an enzyme in the extract, DNA gyrase, to convert relaxed lambda DNA to a superhelical form which could then undergo recombination (26).

Site-specific recombination involving the transposition element $\gamma\delta$ is also dependent on the superhelical state of the DNA (31). Resolvase, an enzyme coded for by $\gamma\delta$, carries out the final step in $\gamma\delta$ transposition only if the DNA is negatively supercoiled.

Superhelicity is also believed to play an important role in homologous genetic recombination (32,33). The ability of duplex DNA to take up homologous single-stranded DNA, forming a recombination intermediate called a heteroduplex joint, is strongly influenced by the superhelical state of the DNA (34). Because of their ability to change the topological linking number of DNA, topoisomerases may also promote homologous recombination between DNA molecules containing no free ends. The ability of type I topoisomerases to catalyze the intertwining of complementary single-stranded rings suggests that they are capable of playing such a role (22,23).

A more direct way in which topoisomerases might promote recombination involves their inherent ability to break and rejoin the DNA backbone, two essential steps in the process of recombination. The first evidence suggesting this type of functional role for topoisomerases came when the lambda integrase protein was discovered to have a type I topoisomerase activity (17). A tantalyzing mechanism by which a nicking-sealing enzyme might promote lambda site-specific recombination has been proposed. The initial step is the formation of a four-stranded DNA structure between specific sites on the lambda and bacterial DNAs. Two molecules of topoisomerase (integrase protein) then bind to and create single-strand breaks at identical sites on strands of the lambda and bacterial DNAs having the same polarity. By a simple rotation of the DNA around these breaks, ends of the lambda DNA can be aligned with the ends of the bacterial DNA. A subsequent topoisomerase mediated resealing of the breaks produces a Holliday structure. The resulting Holliday structure can then be resolved into a fully recombinant molecule by a

final round of breakage and reunion involving the remaining virgin pair of strands (17,35).

Resolvase, the enzyme which catalyzes a site-specific recombination event in γδ transposition, may act by a similar mechanism. It is interesting that resolvase has recently been shown to form covalent complexes with DNA (36) suggesting that it might use a topoisomerase-like mechanism to recombine DNA. A role for topoisomerases in transposition gains additional support from genetic studies. The transposition frequencies of the transposable elements Tn 5, Tn 9, and Tn 10 are dramatically reduced in strains of E. coli deficient in the type I topoisomerase omega protein (37).

Topoisomerases may also promote illegitimate recombination of DNA. Treatment of E. coli with oxolinic acid, a drug which causes DNA gyrase to create double-strand breaks in DNA, results in over a 10-fold increase in the level of illegitimate recombination (38). If cells containing a drug resistant form of gyrase are treated with oxolinic acid, no rise in the level of illegitimate recombination occurs. Eukaryotic type I topoisomerases may also play a role in illegitimate recombination. Evidence for this has come from in vitro studies showing the ability of eukaryotic type I topoisomerases to recombine nonhomologous DNAs (14,39).

General Aim of the Studies

Although the role of topoisomerases in prokaryotic DNA metabolism is becoming clearer, information concerning their cellular role in eukaryotes is almost nonexistent (3). The studies described in the following chapters represent initial efforts to biochemically and

genetically characterize a topoisomerase from the lower eukaryote Ustilago maydis. Such studies will hopefully provide a foundation for probing the role of these enzymes in eukaryotic DNA recombination.

For the sake of continuity, as well as to make the reading easier, the detailed procedures (materials and methods) used in these studies are found in Appendices A - F.

CHAPTER II
PURIFICATION OF PROPERTIES OF THE TOPOISOMERASE

There are a variety of approaches which can be taken in studying recombination. One approach is to reconstruct the events of recombination *in vitro* using purified cellular proteins. Developing an *in vitro* system required actively engaging in purifying proteins which might play an important role in recombination. The purification and characterization of one of these proteins, DNA topoisomerase, are described here.

Development of Novel Filter Assays to Detect Topoisomerase Activity

Due to differences in their tertiary configurations, superhelical and relaxed DNAs can be easily separated by electrophoresis through agarose gels (40). This commonly used technique has provided a simple means for monitoring the conversion of superhelical to relaxed DNA by DNA topoisomerase. However, this assay is time consuming and only semiquantitative. As an alternative, two filter assays were developed which provide a quantitative and rapid determination of topoisomerase activity.

The D-loop filter assay discriminates between superhelical and relaxed DNA by their differing abilities to form stable triple-standard structures called D-loops (41). Superhelical, but not relaxed DNA, can take up homologous single-stranded fragments to form D-loop complexes which are selectively retained on a nitrocellulose filter. Relaxation of superhelical DNA by topoisomerase prevents subsequent D-loop

formation. Therefore a loss of DNA bound to a nitrocellulose filter is considered as a measure of topoisomerase activity.

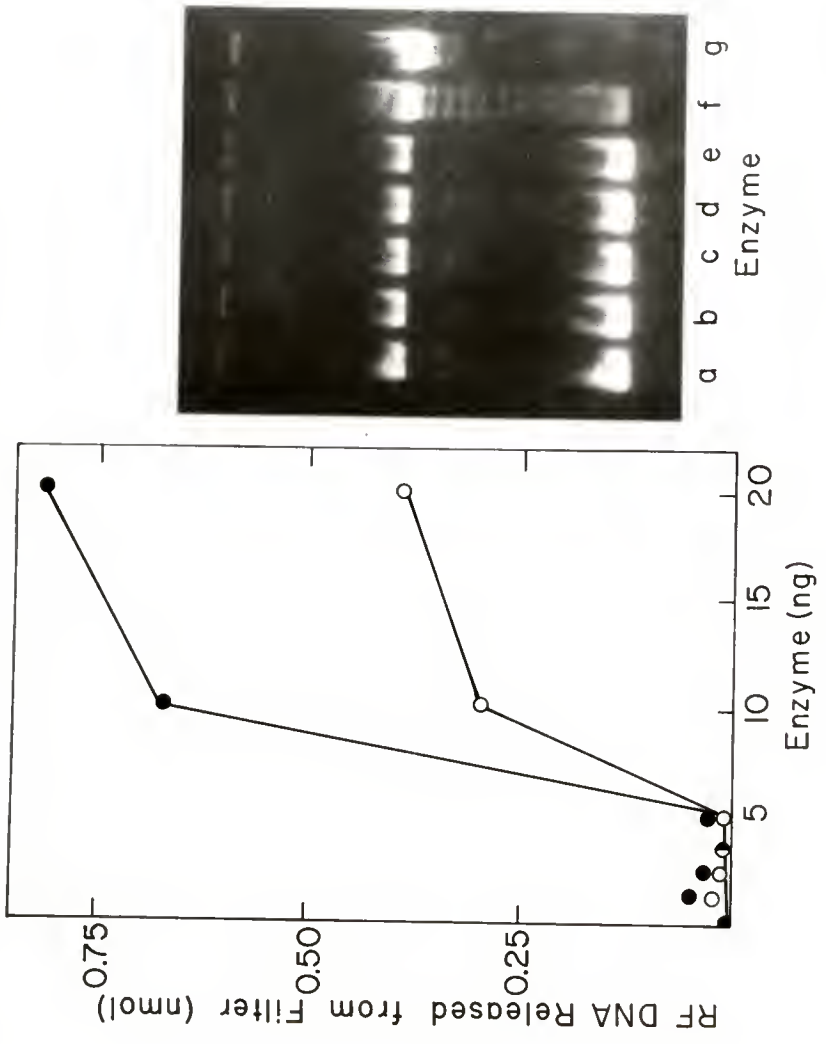
Superhelical and relaxed DNA can also be distinguished by filtration through nitrocellulose under conditions of high ionic strength and this forms the basis of the second filter assay for topoisomerase activity (42). At 3.5 M salt superhelical, but not relaxed DNA, will bind to a nitrocellulose filter. This selective binding may be a result of a structural change in the DNA from a right-handed to a left-handed helix, a transition known to be strongly affected by the superhelical density of DNA as well as the ionic environment (43,44). Superhelical DNA treated with topoisomerase is converted to a relaxed form which is unable to bind to a nitrocellulose filter. Therefore, a loss in DNA binding to a nitrocellulose filter is a measure of topoisomerase activity.

One drawback to both of the filter assays is that neither can differentiate between relaxation activity and endonuclease activity. Fortunately, these two possibilities can be easily distinguished using a nicking assay described by Kuhnlein et al. (45). By using this assay in conjunction with the filter assays, topoisomerase activity can be readily measured.

A comparative measurement of topoisomerase activity using the two filter assays and the gel assay is shown in Figure 1. The loss in DNA binding to a nitrocellulose filter in the two filter assays is seen to be in good agreement with the appearance of a slower moving, relaxed form of DNA present in lanes f and g of the agarose gel. Evident in all three assays is the sigmoidal character of topoisomerase activity, a property exhibited throughout every step in the purification of this enzyme. For

Figure 1. Topoisomerase assays.

Reaction mixtures (100 µl) containing 75 mM potassium phosphate, pH 7.5, 1 mM EDTA, 5% glycerol, and 5 nmol of RF I [^{3}H] DNA (1.5 x 10^4 cpm/nmol), and the indicated amounts of topoisomerase were incubated at 25°C. At the end of 30 min three 25 µl aliquots were removed from each reaction mixture and processed as described in Appendix C. One aliquot was loaded onto a 1.2% agarose gel, the second aliquot was mixed with 1 ml of buffer containing 3.5 M NaCl (○) and the third aliquot was combined with 6.6 nmol of single-stranded DNA fragments and incubated at 75°C for 2 1/2 hr to promote D-loop formation (●). The following amounts of enzyme were used in the lanes of the agarose gel: a, no enzyme; b, 1.25 ng; c, 2.5 ng; d, 3.75 ng; e, 5 ng; f, 10 ng; g, 20 ng.


a b c d e f g
Enzyme
RF DNA Released from Filter (nmol)
0.75
0.50
0.25
5
10
15
20
Enzyme (ng)

this reason, an effort was made to use concentrations of the enzyme within the linear portion of the assay when characterizing a particular reaction parameter.

Isolation of a Topoisomerase with a High Molecular Weight

Due to an instability of the enzyme in low salt buffers and large losses of activity incurred during dialysis, efforts were made during the purification to maintain the enzyme in concentrations of salt above 0.1 M and to avoid dialysis. PMSF was also routinely included in the purification buffers to minimize degradation of the topoisomerase by serine proteases.

The scheme used to purify the topoisomerase is outlined in Table I. Following breakage of the cells in 1 M KCl, polyethylene glycol was added to the extract and the resulting precipitate removed. The supernantant, still containing 1 M salt, was directly applied to a hydroxylapatite column. Activity was eluted using an increasing potassium phosphate gradient in 1 M KCl and loaded directly on to an octyl-Sepharose column equilibrated in 1.0 M KCl. The column was then equilibrated in buffer containing 0.1 M KCl before eluting activity with an increasing gradient of Triton X-100. Relaxing activity was loaded directly onto a phosphocellulose column and subsequently recovered in an increasing KCl gradient. At this stage the enzyme was found to be very stable if stored at -20°C in the presence of 0.1 M KCl and 50% glycerol. Loss of activity under these conditions was less than 10% over a two-year period.

Despite all the precautions taken, the final yield of activity was a mere 0.4%. Although initially disconcerting, this may in fact

TABLE I
PURIFICATION OF THE ENZYME

Fraction	Volume ml	Activity units x 10^{-4}	Protein mg	Specific Activity units/mg
I. Crude Extract	240	920.	2,400.	3,800
II. Polyethyleneglycol	315	315.	583.	5,400
III. Hydroxylapatite	280	560.	15.	37,300
IV. Octyl-Sepharose[a]	85	227.	-	-
V. Phosphocellulose	18	19.2	4.5	42,700
VI. Sephacryl S200[b]	8	3.4	0.40	85,000

[a]Protein determination was not performed since Triton interfered with the assay.

[b]Total amounts indicated are corrected for the fact that only a portion of Fraction V was taken through this step.

NOTE: See Appendix D for details of purification.

represent a loss of an activator of the topoisomerase during the course of purification, a subject which will be returned to later.

The purity of fraction V was tested by electrophoresis through polyacrylamide gels under nondenaturing conditions. Staining of the gel with Coomassie blue revealed two major bands (Fig. 2). The faster mobility band, representing about one-third of the total stained protein, contained the topoisomerase activity. Electrophoresis of this band in a second dimension in a polyacrylamide gel containing SDS revealed a number of bands ranging in size from 2×10^4 to 1.2×10^5 daltons. Not knowing if these multiple bands were degradation products or if they were components of a multi-subunited enzyme, efforts were undertaken to do additional studies designed to determine the native molecular weight of the enzyme. When a portion of fraction V was gel filtered through Sephacryl S-200 in 0.5 M KCl, 45% of the activity was recovered in a peak corresponding to a molecular weight of 270,000 while a small amount, less than 1%, emerged with an apparent size of 30,000 daltons (Table II). This is considerably larger than previously described eukaryotic type I topoisomerases which have molecular weights ranging from 60 - 100,000 (3). Gel filtration in lower salt solutions resulted in a drastic reduction or total loss in activity. The recoverable activity had molecular weights corresponding to 85,000 and 30,000 daltons. No 270,000 dalton form was observed. If the topoisomerase does indeed consist of multiple subunits, low salt conditions may lead to their dissociation.

Figure 2. Polyacrylamide gel electrophoresis of the topoisomerase preparation.

Polyacrylamide gels containing 5% acrylamide, 0.14% N,N'-methylenebisacrylamide, 0.1 M potassium phosphate, pH 7.5, 10% glycerol, were cast in tubes (5 x 80 mm) with ammonium persulfate as catalyst. The gels were prerun overnight with electrode buffer composed of 0.1 M potassium phosphate, pH 7.5, 10% glycerol in order to remove ammonium persulfate. Electrode buffer was replaced, and 100 µl aliquots of the topoisomerase preparation (500 units) containing 50% glycerol and 5 µg of fluorescamine-labeled pancreatic ribonuclease (46) were layered on parallel gels. The samples were overlaid with 100 µl of 20% glycerol and then electrophoresed at 5 mA/tube. Cooling was provided by circulating ice water around the tubes during the course of the run. After 12 h when the ribonuclease marker, located under ultraviolet light, had migrated approximately halfway down the tube, the run was ended. One gel was stained for protein with Coomassie brilliant blue. A parallel gel was cut into 2 mm slices, and each slice was soaked in 100 µl of 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, 0.1 M KCl. After 18 h each slice was assayed for topoisomerase activity by the agarose gel method. Approximately 10% of the activity was recovered after electrophoresis. The rapidly migrating material near slice number 15 is the ribonuclease marker.

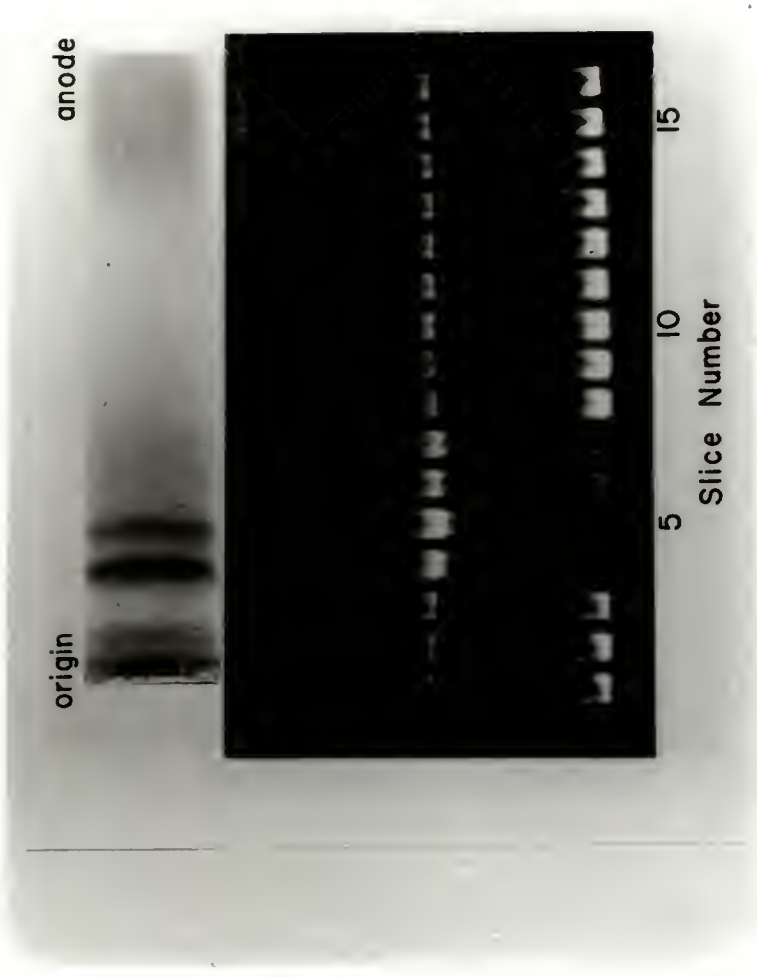

anode
origin
5
10
15
Slice Number

TABLE II
ESTIMATION OF SIZE OF THE TOPOISOMERASE

KCl	Activity Peak	$K_d^{1/3}$	Molecular Weight	Recovery Percent
0.5 M	1	0.54	270,000	45
	2	0.91	30,000	1
0.1 M	1	0.69	85,000	5
	2	0.91	30,000	5
0.05	None			0

NOTE: A column of Sephacryl S-200 (1.4 x 56 cm) was equilibrated with 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, and the indicated salt concentrations. The excluded and included volumes were determined with the use of ^{3}H-labeled $\phi X174$ phage and [^{32}P] orthophosphate, respectively. Marker proteins used to calibrate the column included ferritin, catalase, bovine serum albumin, ovalbumin, pancreatic deoxyribonuclease, and cytochrome c. The hydrodynamic distribution coefficent K_d was determined (47) and linearly related to molecular weight (48) by assuming the topoisomerase is a globular protein. Topoisomerase (7000 units) was loaded, and the column was run at 10 ml/hr. Fractions of 1 ml each were collected and assayed for activity by the agarose gel assay. Recovery of activity was estimated by visual inspection of the gels.

Sedimentation analysis was also used to determine the size of the native enzyme. As shown in Figure 3, activity was present in a single peak with a sedimentation coefficient of 2.5 S corresponding to a spherical shaped protein of 29,000 daltons. Although a variety of buffers and salt conditions were used, recovery of activity was poor, less than 1%. Even presoaking the centrifuge tubes in solutions of BSA did not improve recovery. This loss of activity is puzzling especially since the topoisomerase should be stable in the solutions that were used. Possibly the enzyme was affected by the high pressures generated during the centrifugation (49).

A variety of other purification schemes were also attempted in an effort to improve upon the specific activity of the enzyme but the specific activities obtained were never much higher than the value of 8.5×10^4 for fraction VI.

Examination of Parameters Affecting Enzymatic Activity

As is illustrated in Figure 4, relaxation activity spans a pH range from 5.5 - 8.0 being acutely sensitive to the buffer used. While high activity was observed at pH 7.5 in phosphate buffer, activity was halved in Tris-HCl and not measurable in either 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid or imidizole buffer. Salt also affected activity as is shown in Figure 5. There was a sharp response in activity as the salt concentration increased with optimal stimulation occurring around 0.2 M KCl. Although as much as a 5-fold increase in activity could be seen with KCl, Mg^{2+} proved to be much more effective in promoting activity. In the presence of 10 mM $MgCl_2$,

Figure 3. Centrifugation of topoisomerase in a glycerol gradient.

Topoisomerase (500 units) together with 1 mg of bovine serum albumin (BSA) and 10 µg of catalase in 0.1 ml was layered on top of a 5 ml 20 - 40% gradient of glycerol containing 20 mM potassium phosphate, pH 7.0, 5 mM 2-mercaptoethanol, 0.5 M KCl. Centrifugation was at 4°C for 22 h at 50,000 rpm in the Beckman SW 50.1 rotor. At the end of the run fractions were collected from the bottom of the gradient and assayed for topoisomerase activity by the agarose gel assay. Activity was determined by visual inspection, and recovery was estimated at less than 1%. Catalase activity was located by assay and bovine serum albumin by protein determination; peaks are indicated in arbitrary units. The sedimentation coefficient and molecular weight of the topoisomerase were determined according to Martin and Ames (50). Sedimentation is from right to left.

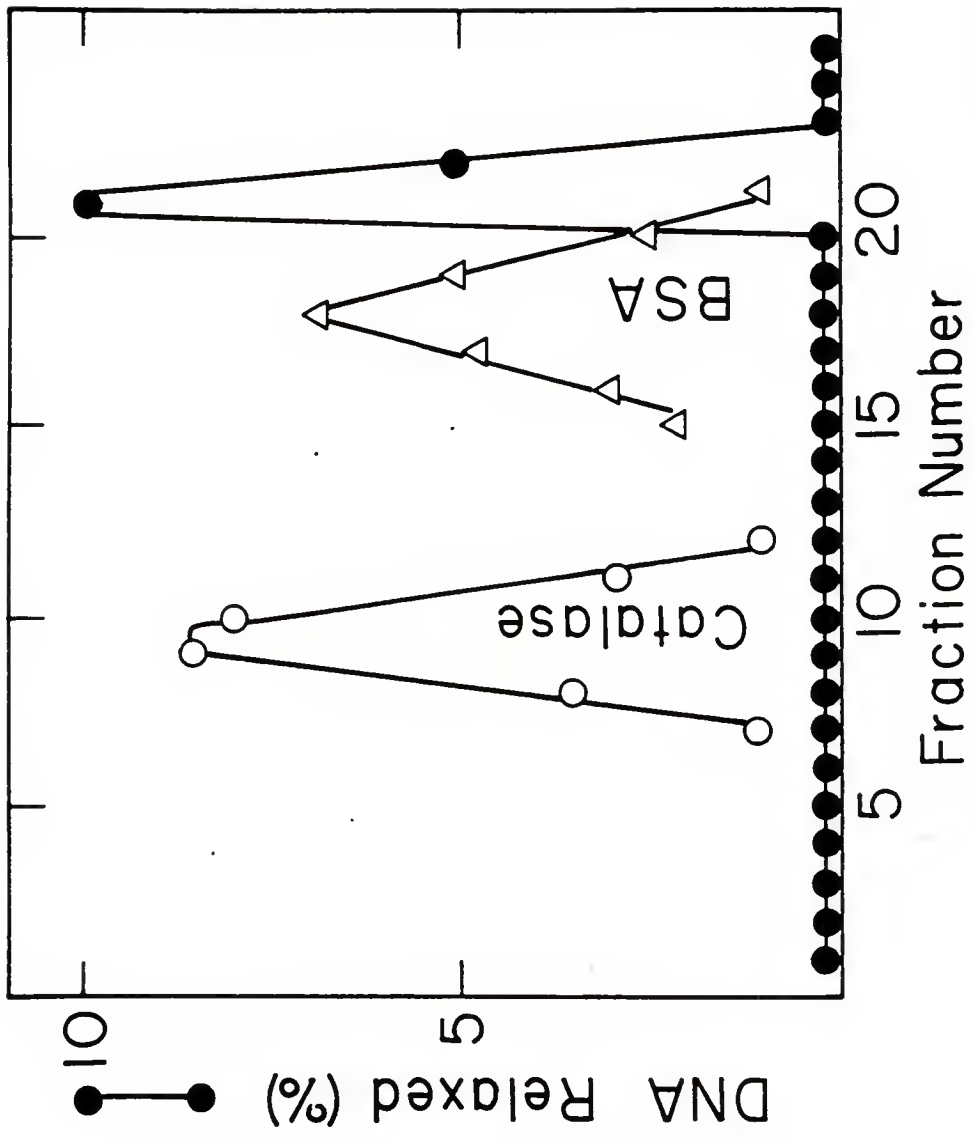

DNA Relaxed (%)
10
5
5
10
15
20
Fraction Number
BSA
Catalase

Figure 4. pH optima of the topoisomerase.

Reaction mixtures (50 µl) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 3 nmol ϕX RF I [^{3}H] DNA (1.5×10^4 cpm/nmol), 1 unit of topoisomerase and 20 mM sodium citrate (□), Tris-HCl (△), potassium phosphate (○), HEPES (●), or Imidizole (x) were incubated at 37°C for 30 min. Reactions were stopped by adding 2.5 µl 10% SDS and 10 µl 3 M NaCl. Each reaction was then split into two 30 µl aliquots and processed as described in Appendix C. One aliquot was used to assess nicking activity. The pH of the second aliquot was adjusted by adding 3 µl of 0.5 M Tris-HCl, pH 7.5 before adding 5 nmol of single-stranded DNA fragments. After heating at 75°C for 3 h, the amount of D-loop formation was determined as described in Appendix C.

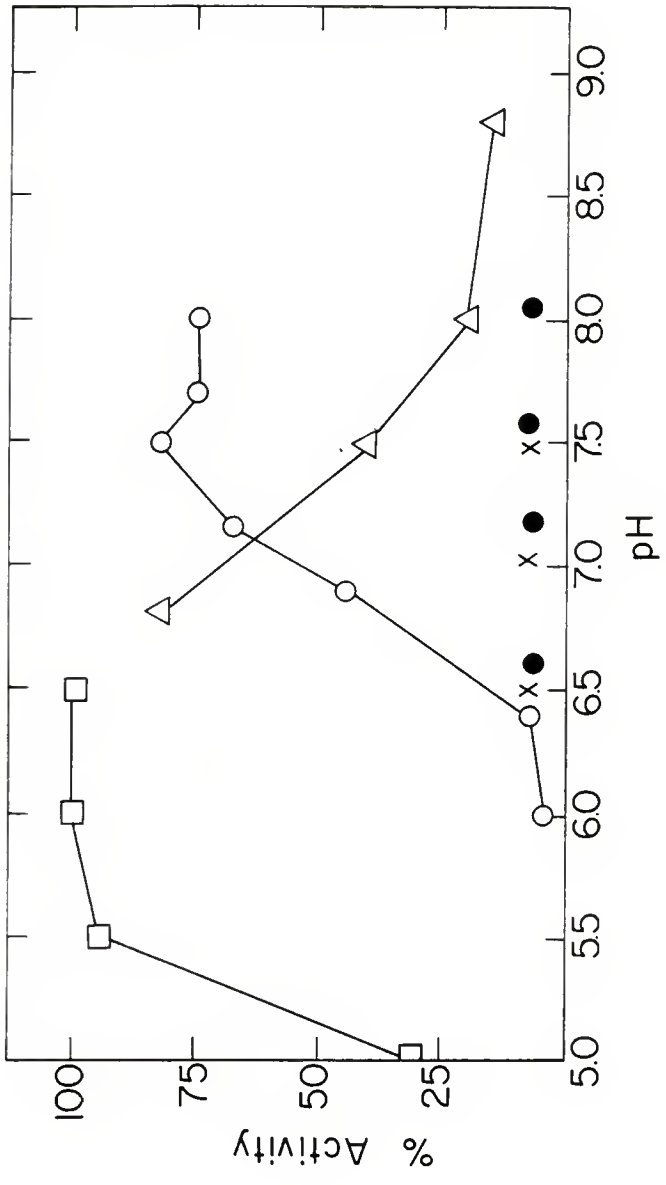
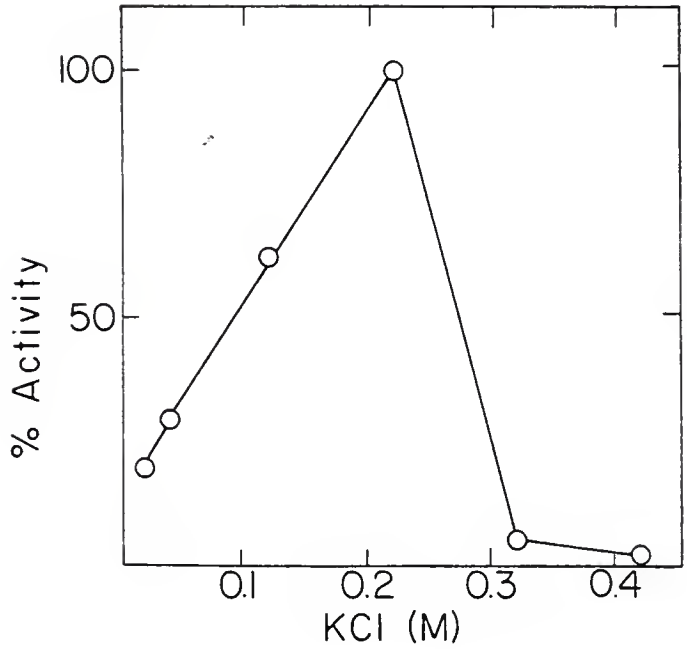

% Activity
100
75
50
25
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

Figure 5. Salt optima of the topoisomerase.

Reaction mixtures (25 µl) containing 25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.4 mM 2-mercaptoethanol, 3 nmol ϕX RF I [^{3}H] DNA (1.5 x 10^4 cpm/nmol), 100 ng topoisomerase and various concentrations of KCl were incubated at 25°C for 30 min. Reactions were terminated by adding 1 ml of buffer containing 3.5 M NaCl and topoisomerase activity determined using the high salt filter assay as described in Appendix C.

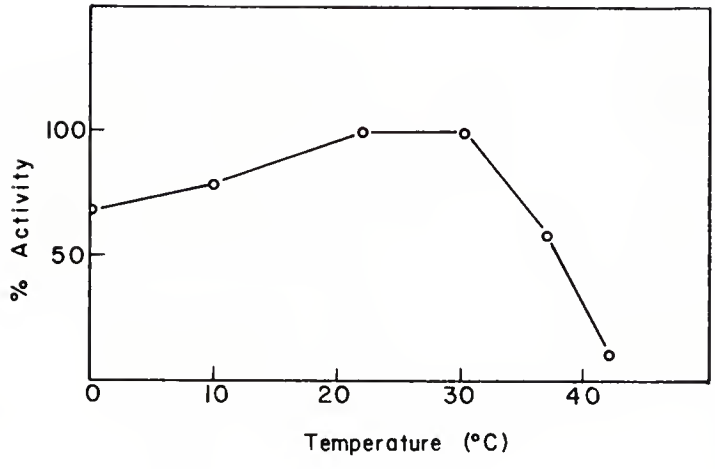

100
50
% Activity
0.1
0.2
0.3
0.4
KCl (M)

relaxation of the DNA was enhanced 25-fold. The effect of other divalent cations on topoisomerase activity was also tested with curious results. In contrast to Mg^{2+}, Cu^{2+} and Zn^{2+} were strong inhibitors of the topoisomerase. The presence of as little as 20 μM $CuCl_2$ or $ZnCl_2$ totally inhibited activity. Whether this is due to a direct interaction of these metal ions with the topoisomerase or if it is a result of an interaction with the DNA is unclear. Topoisomerase was active in the presence of the chelating agents EDTA, 8-hydroxyquinoline, diethyldithiocarbamic acid and isoniazed but was potently inhibited by 1,10 phenanthroline. The inhibition does not apparently involve the chelation of an enzyme-bound metal since the nonchelating analog 4,7 phenanthroline also inhibited activity. The optimal temperature for topoisomerase activity was found to be between 20 - 30°C with no detectable activity at 42°C (Fig. 6). Surprisingly, the enzyme is fairly active at low temperatures with 70% relaxation occurring at 0°C. A reducing agent is necessary to maintain activity. Storage of the enzyme in the absence of 2-mercaptoethanol resulted in a loss of activity. However, restoration of activity could be seen upon the addition of fresh reducing agent to the reaction mixture. In addition, the inclusion of 2 mM N-ethylmaleimide to a reaction containing 0.1 mM 2-mercaptoethanol inactivated the enzyme.

As was discussed in Chapter I, eukaryotic type I topoisomerases catalytically remove superhelical turns in the absence of an added energy cofactor (7). This is in contrast to eukaryotic type II enzymes whose catalytic action on DNA is coupled with the hydrolysis of ATP

Figure 6. Temperature optima of the topoisomerase.

Reactions (50 µl) containing 75 mM potassium phosphate, pH 7.5, 10% glycerol, 3 nmol ϕXRF I [^{3}H] DNA (1.5 x 10^4 cpm/nmol) and 40 ng of topoisomerase were incubated for 30 min at the indicated temperatures. Reactions were terminated by adding 10 µl 3 M KCl. Topoisomerase activity was then determined using the D-loop assay as described in Appendix C.


100
50
% Activity
0
10
20
30
40
Temperature (°C)

(3). Similar to type I enzymes, the Ustilago topoisomerase relaxes DNA in the absence of an energy cofactor. However, curiosity led us to investigate if ATP had any effect on the Ustilago topoisomerase. At 5 mM, ATP totally blocked activity (Table III). This inhibition was not simply due to chelation of an endogenous metal essential to topoisomerase activity since EDTA at the same concentration exhibited no effect on the action of the enzyme.

Is hydrolysis of ATP necessary for inhibition? Several different nonhydrolyzable analogs of ATP including α,β-methylene ATP, β,γ-methylene ATP, adenyl-5'-imidodiphosphate, and adenosine-5'-O-(3-thiotriphosphate) were tested and all of them totally inhibited the topoisomerase at 5 mM suggesting that hydrolysis was not required for inhibition (Table III). In addition, attempts were made to measure ATPase activity and no significant hydrolysis was detected in standard reaction mixtures containing [γ-^{32}P] ATP and superhelical DNA. Inhibition is nucleotide specific. None of the other nucleotide triphosphates tested inhibited the topoisomerase at 5 mM. Although slight inhibition of activity was caused by ADP, AMP was without effect confining the inhibition to the triphosphate form of adenosine.

Inhibition is probably the result of a direct interaction of ATP with a site on the topoisomerase. Efforts to dissociate the ATP inhibition from the topoisomerase by electrophoresis of the enzyme through a polyacrylamide gel under nondenaturing conditions were unsuccessful. The topoisomerase activity associated with the faster moving band in Figure 2 was still found to be blocked by ATP. In addition, topoisomerase isolated using a number of different purification schemes was always

TABLE III
INHIBITION BY NUCLEOTIDES AND POLYNUCLEOTIDES

Compound	Concentration mM	Activity Percent
Control		100
ATP	5.0	0
ADP	5.0	70
AMP	5.0	100
dATP	5.0	90
α,β-methylene ATP	1.6	50
α,β-methylene ATP	3.2	0
β,γ-methylene ATP	1.5	100
β,γ-methylene ATP	3.0	0
AMP-PNP[a]	5.0	0
ATP-γ-S[a]	5.0	0
CTP	5.0	100
GTP	5.0	100
TTP	5.0	100
UTP	5.0	100
dCTP	5.0	100
dGTP	5.0	100
Poly(rA)	0.02	100
Poly(dA)	0.02	100
Poly(dC)	0.02	100
Poly(dT)	0.02	100
Poly(dG)	0.01	0
Poly(d(G-C))	0.02	100
φχ174 DNA	0.2	50
P22 DNA	0.16	100

[a]AMP-PNP, adenyl-5'-imidodiphosphate.

[b]ATP-γ-S, adenosine-5'-O-(3-thiotriphosphate).

NOTE: Reaction mixtures containing the indicated concentrations of compounds were run at standard conditions and activity was estimated by the agarose gel assay. Because of the narrow range where activity increases with added enzyme, several enzyme concentrations were used in testing each compound to ensure that the response was in the linear range of the assay. All the results presented are relative to a control where approximately 90% of the substrate DNA was relaxed. It should be emphasized that these results are only approximate since activity was estimated by visual inspection of the gels.

found to be sensitive to ATP, again implying the inhibition results from an interaction of ATP with part of the topoisomerase. Whether this inhibition involves a blockage of the enzyme's active site, or is a result of an allosteric change mediated by the binding of ATP to a region other than the active site, is unclear. Regardless, inhibition of the topoisomerase by ATP may be a key to understanding how this enzyme is regulated in the cell.

Several type I topoisomerases are sensitive to polynucleotides. Single-stranded DNA effectively inhibits the E. coli omega protein (11), calf thymus nicking-closing enzyme (51), and the bacteriophage lambda integrase gene topoisomerase (17). The Ustilago topoisomerase is also inhibited by single-stranded DNA but is more acutely sensitive to the homopolymer poly(dG) (Table III). Other polymers tested, including poly(d(G-C)), were ineffective inhibitors at comparable concentrations. Possibly the unusual secondary structure of poly(dG) is responsible for this selective inhibition (52).

Antibiotic Inhibitors

Antibiotics can serve as powerful tools for elucidating the cellular role of proteins. The sensitivity of DNA gyrase to the drugs nalidixic acid and novobiocin was utilized to establish the regulatory role of this topoisomerase in prokaryotic replication, transcription and recombination (3). The Ustilago enzyme is also inhibited by nalidixic acid and novobiocin. The rate of relaxation by the Ustilago topoisomerase is reduced approximately 2-fold in reactions containing 20 μg/ml of nalidixic acid (Table IV). This is a 10-fold lower concentration

TABLE IV
INHIBITION BY ANTIBIOTICS

Drug	Concentration µg/ml	Activity Percent
Control		100
Novobiocin	100.0	30
	200.0	0
Nalidixic acid	20.0	50
Berenil	2.8	50
	5.6	10

NOTE: Reaction mixtures containing the indicated concentrations of antibiotic were assayed under standard conditions as described in Table III.

than is required to cause a similar inhibition of DNA gyrase (53). While 200 μg/ml of novobiocin are required to totally inhibit the Ustilago enzyme, significant loss of activity is seen at 100 μg/ml. Berenil, an antitrypanosomal drug previously shown to inhibit a mitochondrial topoisomerase, also inhibits the Ustilago enzyme (54). Half-inhibition of topoisomerase activity occurs at 5.6 μg/ml.

Comparison of Superhelical Substrates

Like other eukaryotic topoisomerases, the Ustilago enzyme can remove positive as well as negative supercoils from DNA. To create a positively supercoiled substrate, ethidium bromide was added to a reaction containing closed circular relaxed DNA. If the topoisomerase acts upon this substrate, subsequent removal of both the enzyme and the ethidium bromide should result in a negatively superhelical molecule of DNA which can be easily distinguished from the starting material by electrophoresis through an agarose gel. The presence of a faster moving band in Figure 7 indicates an ability of the topoisomerase to remove positive superhelical turns from DNA.

Are positive and negative supercoils equally good substrates for the topoisomerase? A comparison was made using the high salt filter assay. Although positive superhelical DNA was relaxed at a much slower rate than negatively superhelical DNA, there was no longer a sigmoidal dependence of activity on the enzyme concentration (Fig. 8). Although this difference in reaction kinetics may simply be an artifact of using ethidium bromide to generate positive superhelical DNA, it might alternatively represent the action of topoisomerase on two different types of structural elements in the DNA.

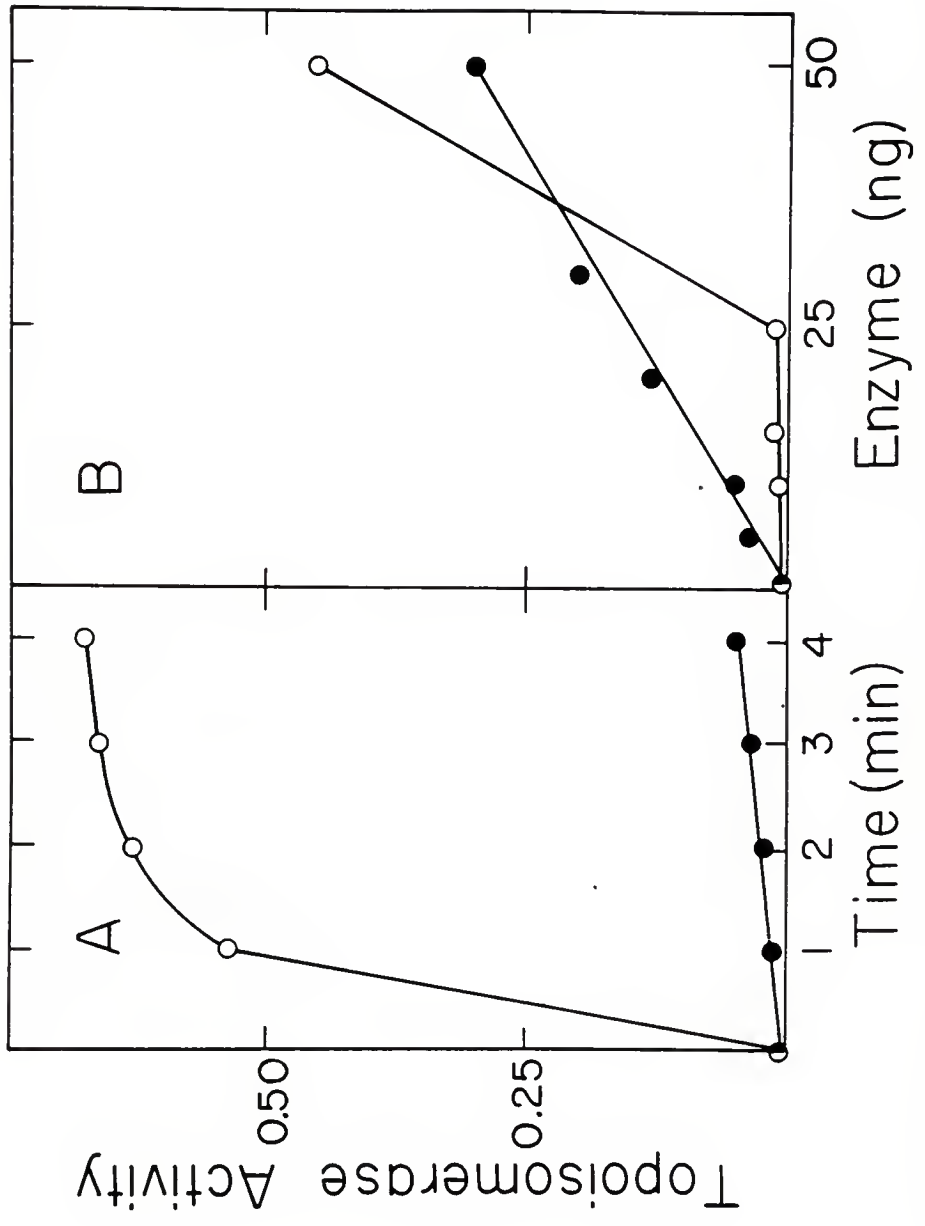
Figure 7. Removal of positive superhelical turns--gel assay.

Reaction mixtures (50 µl) containing 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 5% glycerol, 0.8 µg/ml ethidium bromide and 3 nmoles ϕχ RF I or RF IV DNA were incubated in the presence or absence of topoisomerase at 25°C. After 30 min the ethidium bromide and protein were removed by extracting with 1 volume of phenol. The DNA was then loaded onto a 1.2% agarose gel. (Lane A) ϕχRF I DNA; (Lane B) ϕχRF IV DNA; (Lane C) ϕχRF IV DNA and 100 ng topoisomerase.

A B C

Fig. 8. Removal of positive superhelical turns.

The high salt binding assay was used for measuring removal of both negative and positive superhelical turns. However, in the latter case an increase, rather than decrease, in the amount of DNA bound to filters is a measure of removal of positive supercoils. When form I DNA alone was washed through a nitrocellulose filter, as described, approximately 70% of the input radioactivity bound to the filter. When form IV DNA was tested, 2% bound. If ethidium bromide was present there was no need to extract it from the DNA as long as the solution was diluted sufficiently before filtering. Again less than 2% of the radioactivity bound to a filter if a solution containing form IV DNA and 1 μg/ml of ethidium bromide was diluted 1 to 40 in 3.5 M NaCl before filtering. A, rate of reaction. Two reaction mixtures (150 μl) were set up under the standard condition, one containing 7.5 nmol of ϕχRF I[^{3}H] DNA (2.2 x 10^4 cpm/nmol) and the other containing 7.5 nmol of ϕχRF IV [^{3}H] DNA (2.2 x 10^4 cpm/nmol) and 1 μg/ml of ethidium bromide. Topoisomerase (300 ng) was added to each reaction, and the mixtures were incubated at 25°C. AT the indicated times aliquots of 25 μl were removed, diluted into 1 ml of the 3.5 M NaCl buffer for binding, and processed as described. After 30 min of reaction the amount of DNA bound to a filter after removal of positive superhelical turns reached a maximum of 65% of the input form IV DNA. B, enzyme concentration curve. Reaction mixtures (25 μl) containing 1.25 nmol of ϕχRF I [^{3}H] DNA or ϕχRF IV [^{3}H] DNA plus 1 μg/ml of ethidium bromide and the indicated amounts of topoisomerase were incubated at 25°C for 30 min and then filtered as above. (○), negative superhelical DNA; (●), positive superhelical DNA.


Topoisomerase Activity
0.50
0.25
A
B
1
2
3
4
Time (min)
25
50
Enzyme (ng)

Activity is Stimulated by H1 Histone

Histone H1 shows a specific interaction with superhelical DNA (55). This tantalyzing property led to a search for a possible interrelationship between H1 histone and the Ustilago topoisomerase. The dramatic effect of H1 on topoisomerase activity is illustrated in Figure 9. In the presence of H1, topoisomerase activity is stimulated more than 25-fold. Although the ratio of H1 to DNA used in Figure 9 was 0.025 (w/w), a ratio of 0.01 (w/w) or 1.4 H1 molecules per molecule of superhelical DNA was equally as effective in stimulating the topoisomerase. The ratio of H1 to DNA is important; a 10-fold increase or decrease relative to 0.01 (w/w) abolishes the stimulatory effect. In agreement with Bina-Stein and Singer, high ratios of H1 to DNA, above 0.2 (w/w), inhibit topoisomerase activity (56). In contrast to H1, none of the other histones, poly L-lysine, or spermidine proved to be as effective in stimulating topoisomerase activity (Fig. 10).

Although the H1 histone used in these studies was greater than 90% pure, as judged from SDS polyacrylamide gels, the possibility existed that stimulation of the topoisomerase was due to a contaminating protein present in the H1 preparation. To free H1 of contaminants it was electrophoresed through a 15% SDS polyacrylamide gel. The gel was stained with Coomassie blue and the two major staining bands, containing different structural species of H1 (57,58) were separately cut out and the protein extracted. Addition of protein from either of these bands to reaction mixtures stimulated the topoisomerase greater than 10-fold (Fig. 11), providing additional evidence that H1 histone is responsible for stimulation of the topoisomerase. This result also implies that

Figure 9. Stimulation of topoisomerase activity by histone H1.

Reaction mixtures (25 µl) containing 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10% glycerol, 3 nmol of $\phi\chi$ RF I [^{3}H] DNA (1.5×10^4 cpm/nmol), the indicated amounts of topoisomerase, and either 1 µg/ml of histone H1 (○) or 1 µg/ml of bovine serum albumin (●) were incubated at 25°C. After 30 min EDTA was added to a final concentration of 15 mM, and SDS was added to 0.5%. The mixture was diluted with 3 ml of 3.5 M NaCl buffer and filtered through nitrocellulose as described in Appendix C. It was important to dilute the sample 100-fold before filtering to prevent interference by the SDS.

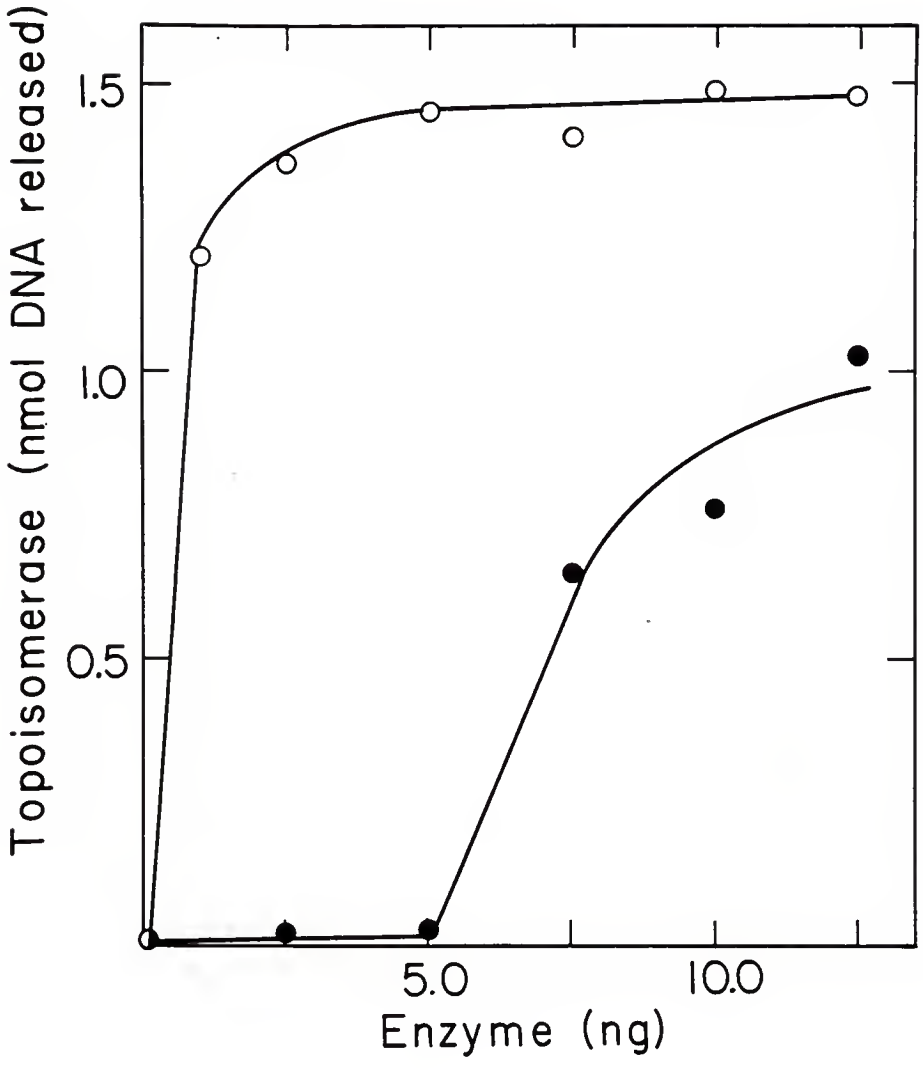

Topoisomerase (nmol DNA released)
1.5
1.0
0.5
5.0
10.0
Enzyme (ng)

Figure 10. Stimulation of topoisomerase activity by histones and polyamines.

Reaction mixtures (25 µl) containing 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl^2$, 10% glycerol, 3 nmol of $\phi\chi$RF I [^{3}H] DNA, and the following additions were incubated at 25°C for 30 min. Reactions were terminated by adding 5 µl of 2% SDS in 0.12 M EDTA and proteinase K to 100 µg/ml. After 30 min at 37°C samples were loaded and run on an agarose gel as described in Appendix C. (a) 10 ng of topoisomerase; (b) 20 ng of topoisomerase; (c) 50 ng of topoisomerase; (d) 10 ng of topoisomerase, 1 µg/ml of H1; (e) 20 ng of topoisomerase, 1 µg/ml of H1; (f) 10 ng of topoisomerase, 1 µg/ml of H2a; (g) 20 ng of topoisomerase, 1 µg/ml of H2a; (h) 10 ng of topoisomerase, 1 µg/ml of H2b; (i) 20 ng of topoisomerase, 1 µg/ml of H2b; (j) 10 ng of topoisomerase, 1 µg/ml of H3 and H4; (k) 20 ng of topoisomerase, 1 µg/ml of H3 and H4; (1), 10 ng of topoisomerase, 1 µg/ml of poly-L-lysine; (m) 20 ng of topoisomerase, 1 µg/ml of poly-L-lysine; (n) 10 ng of topoisomerase, 2 mM spermidine; (o) 20 ng of topoisomerase, 2 mM spermidine; (p) no enzyme.

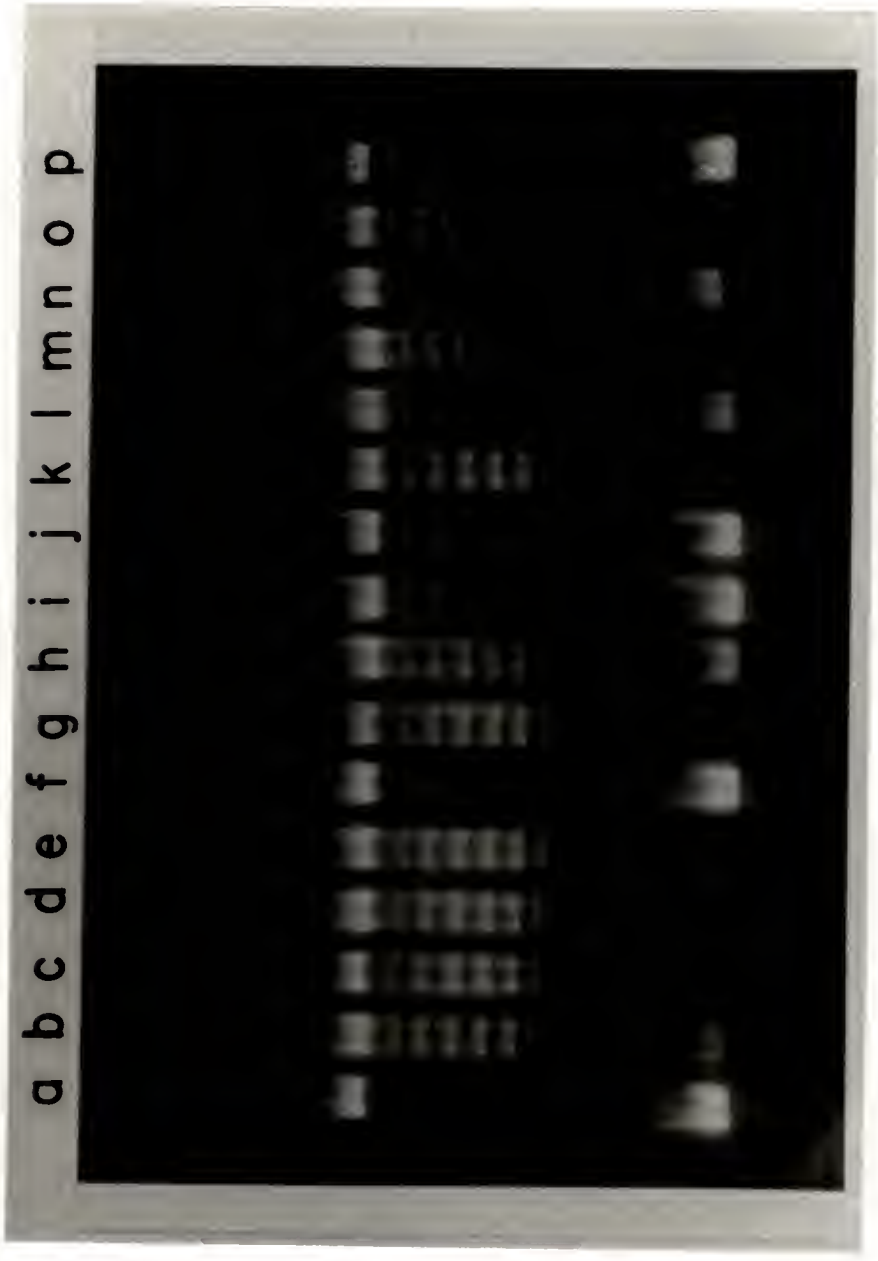

a b c d e f g h i j k l m n o p

Figure 11. Stimulation of topoisomerase by electrophoretically purified H1 histone.

H1 histone (50 μg) was separated from contaminating protein by electrophoresis through a 15% SDS polyacrylamide gel as described in Appendix E. Following electrophoresis the gel was stained with Coomassie blue to visualize the protein. H1 histone was present as a doublet and represented greater than 90% of the stained protein. The fast and slow moving bands of the doublet, which represent different structural forms of H1 (57,58), stained with approximately equal intensity. Each of these bands was excised from the gel and separately dounce homogenized in 500 μl of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl and 0.1% SDS. After the suspensions were allowed to stand at room temperature for 1 hr they were briefly centrifuged to remove the gel debris. To the resulting supernatants was added 125 μl of 100% TCA (final concentration 20%). After standing for 1 hr at -20°C, the protein was pelleted by centrifuging in an Eppendorf Microfuge for 5 min at 4°C. The resulting pellets were washed 1 time with 500 μl acid acetone (1/100 dilution of concentrated HCl in acetone) and then 2 times with 500 μl of acetone. After removing the residual acetone under vacuumn, the fast and slow mobility forms of H1 were each resuspended in 100 μl of 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA. To determine stimulation of the topoisomerase a 1:2.5 or 1:10 dilution of the gel extracted histone was made into reactions (25 μl containing 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.4 mM 2-mercaptoethanol, 5% glycerol, 10 μg/ml BSA, 3 nmol ϕχ RF I [^{3}H] DNA, + topoisomerase. After 30 min at 25 °C, the reactions were terminated by adding EDTA to 15 mM and SDS to 0.5%. The products of the reactions were then analyzed by electrophoresis as described in Appendix C. A, 1.7 ng topoisomerase; B, 8.5 ng topoisomerase; C, 1.7 ng topoisomerase, fast mobility form of H1 histone (1:10); D, 1.7 ng topoisomerase, fast mobility form of H1 histone (1:2.5); E, 1.7 ng topoisomerase, slow mobility form of H1 histone (1:10); F, 1.7 ng topoisomerase, slow mobility form of H1 histone (1:2.5); G, fast mobility form of H1 histone (1:2.5); H, slow mobility form of H1 histone (1:2.5); I, no topoisomerase or H1 histone.

Topo
HI lower band
HI upper band
Controls
A B C D E F G H I

more than one structural form of H1 has an ability to stimulate topoisomerase activity.

The effect of H1 on topoisomerase activity is not entirely surprising. Histone H1 has been previously reported to copurify with type I topoisomerases from calf thymus and mouse L cells (59,60). Other studies have shown that H1 histone can substitute for a cellular protein in promoting catenation of duplex DNA by type II topoisomerases from Drosophila and yeast (61,62). Possibly H1, or an H1-like protein, makes up an integral part of the Ustilago topoisomerase. Separation of this H1-like component from the nicking-closing component of the topoisomerase during purification might explain the low yields of activity as well as the inability to completely purify the enzyme. If H1 is indeed complementing a component normally present in the topoisomerase, this may provide clues about the mechanism and regulation of topoisomerase activity in the cell.

Although a direct interaction of H1 with the topoisomerase may be responsible for stimulating relaxation of the DNA, other possibilities should be considered. As was mentioned earlier, H1 histone has a specific ability to interact with superhelical DNA (55,63). Bina-Stein and Singer have suggested this specificity is related to the binding of H1 at crossover points in the DNA (56). These crossover points occur at a much higher frequency in a DNA molecule with superhelical character (64). Because of its cationic character, H1 may act to neutralize the high negative charge density which would occur at a DNA crossover and thus serve to stabilize an association between two duplex strands of DNA. Perhaps the topoisomerase acts at a crossover structure in the

DNA. An ability to stabilize this structure may account for the ability of H1 to stimulate topoisomerase activity.

Binding of H1 at crossover points in the DNA has received additional support from chromatin studies. Based on electron microscopy and nuclease digestion data, binding of H1 to chromatin causes the DNA to enter and exit a nucleosome at the same site resulting in a crossover structure (64,65). This result is interesting in light of recent studies which show the ability of DNA gyrase to form a nucleosome-like structure with DNA (66). The ability to form similar types of structural complexes with DNA maybe a property common to all topoisomerases. If so, H1 or H1-like proteins may play an important role in regulating topoisomerase activity because of their ability to interact with such complexes.

Structures in the DNA other than a crossover, such as a left-handed Z-helix (43,67), might also be stabilized by H1 and serve as a site for topoisomerase action. The formation of a Z-configuration in alternating d(pCpG) sequences has been shown to be facilitated by salt or by the DNA being negatively supercoiled (44,68). Because of its high charge, H1 may be able to substitute for the high concentrations of salt ordinarily required to induce DNA into a Z-form. This idea is supported by the observation that a large change in conformation, as monitored by a partial inversion of the circular dichroism spectrum, accompanies the binding of H1 to DNA (69) at the moderate salt concentrations (0.14 M) where topoisomerase activity is high. When the alternating sequence d(pCpG) undergoes a salt-induced transition from a β-form to a Z-form, an even more dramatic change takes place as is evidenced by a full

inversion of the spectrum. Possibly binding of H1 to superhelical DNA helps to stabilize short stretches of DNA in the Z-form providing a target for topoisomerase action.

CHAPTER III
PURIFICATION AND PROPERTIES OF H1-LIKE PROTEINS FROM *Ustilago maydis*

The effect of H1 histone on the ability of the *Ustilago* topoisomerase to relax superhelical DNA may represent an important mechanism for controlling DNA structure. However, because the H1 histone used to stimulate the topoisomerase had been obtained from calf thymus, some doubt remained as to whether a protein with properties similar to H1 histone existed in *Ustilago*. The results of the investigator's efforts to identify an H1-like protein in *Ustilago* are presented in this chapter.

An Endogenous Factor in the Topoisomerase Preparation Stimulates Relaxing Activity

Greater than 99% of the topoisomerase activity present in crude extracts of *Ustilago* was lost during the course of enzyme purification (refer to Table I). In contrast, Vosberg and Vinograd found they could obtain good yields of highly purified topoisomerase from LA9 cells, rat liver and calf thymus (70). Interestingly, the major polypeptide present in their highly purified enzyme was H1 histone. If H1 histone was necessary for good topoisomerase activity, a separation of H1 or an H1-like protein from the *Ustilago* topoisomerase during the purification procedure might have accounted for the high losses in activity. Such a separation may have been responsible for the large loss in activity which resulted when the topoisomerase was chromatographed on phosphocellulose. An effort was therefore made to determine if H1 histone was present in the

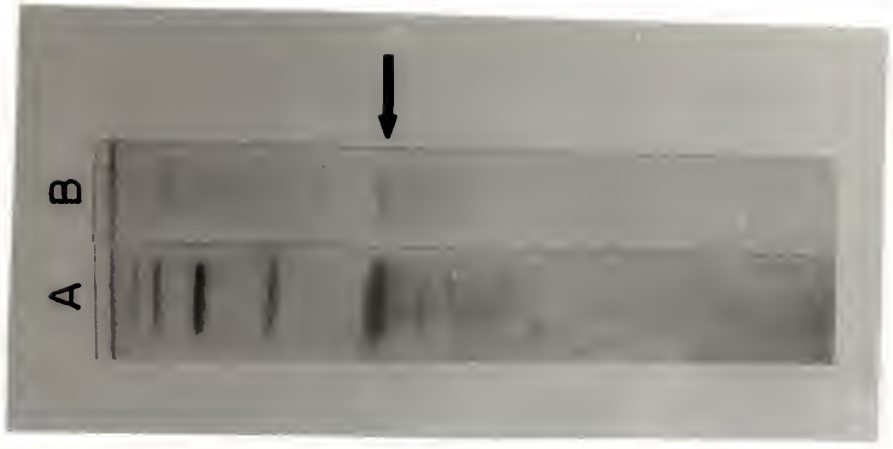
enzyme fraction prior to phosphocellulose chromatography. Because of its high lysine content, H1 histone can be easily purified from most other proteins by its ability to remain soluble in 5% perchloric acid (71). When an aliquot from fraction IV of the topoisomerase purification was extracted with 5% perchloric acid, the acid soluble portion was found to contain a component which stimulated topoisomerase activity (Fig. 12). By itself, the acid-soluble fraction contains no topoisomerase activity. Although maximal stimulation of the topoisomerase by the acid-soluble fraction occurred at pH 6.0 in sodium acetate buffer, good stimulation was also observed at pH 7.5 in Tris-HCl. Electrophoresis of this fraction through an SDS polyacrylamide gel revealed a faintly staining band with a corresponding molecular weight of 20,000 (Fig. 12 B). However, the low amount of protein loaded onto the gel made it difficult to detect minor protein species. Therefore, it was unclear if the 20,000 dalton protein was responsible for stimulating the topoisomerase.

Purification of an H1-like Protein from Ustilago

The stimulation of the *Ustilago* topoisomerase by an endogenous acid-soluble component suggested the presence of an H1-like protein in *Ustilago*. Acid extraction procedures have been successfully used to isolate H1-like proteins from several lower eukaryotes (72-76). Several laboratories have isolated an H1-like protein from *Saccharomyces* by extracting with 5% perchloric acid (72,73). Using a modification of this procedure, it was possible to isolate an H1-like protein from *Ustilago*. The scheme used to isolate these proteins is outlined in Figure 13. The sulfhydral reagents p-chloromercuriphenyl sulfonate and 5,5'-dithiobis (2-nitrobenzoate) were initially included in the extraction buffers

Figure 12. An acid-soluble protein from the topoisomerase preparation stimulates relaxing activity.

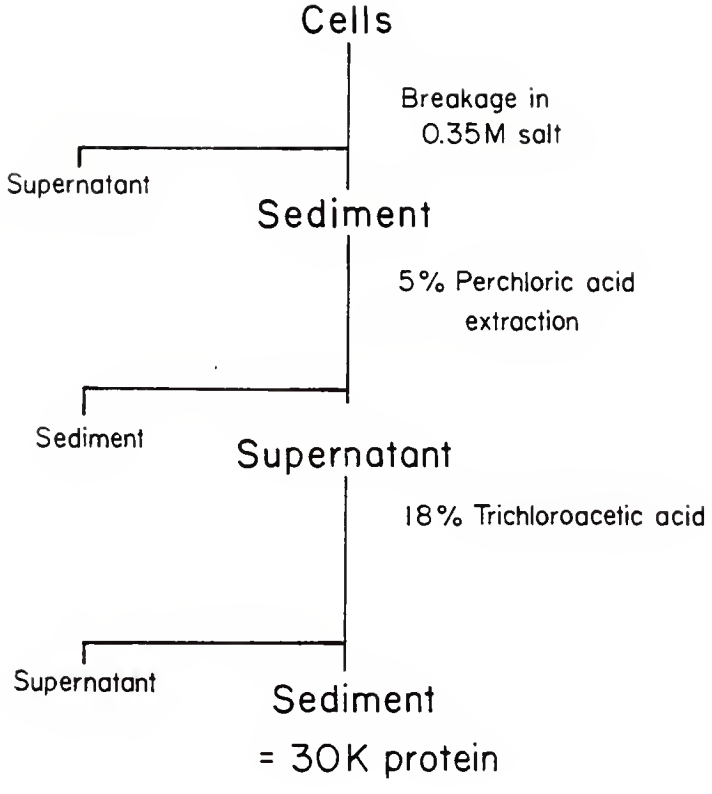
To obtain the acid-soluble protein, 38.5 µl of 70% perchloric acid were added to 500 µl of fraction IV of the topoisomerase purification. After standing for 10 min the sample was centrifuged for 5 min in an Eppendorf microfuge. The pellet was discarded and to the supernatant was added 167 µl of 100% trichloroacetic acid. After standing 10 min the sample was centrifuged and the resulting supernatant discarded. The pellet was washed 1 time with 500 µl, acid-acetone (0.1 ml concentrated HCl in 10 ml acetone) and 2 times with 500 µl of acetone. After removing the residual acetone under vacuum, the pellet was resuspended in 50 µl of 25 mM Tris-HCl, pH 7.5. Part A: Stimulation of the topoisomerase by the acid-soluble protein. Reaction mixtures (25 µl) containing 10 mM $MgCl_2$, 0.4 mM 2-mercaptoethanol, 0.4 mM potassium phosphate, pH 7.5, 5% glycerol, 50 µg/ml BSA, 2.1 nmol $\phi\chi$ RF I DNA, and the following additions were incubated at 25°C. After 30 min the reactions were stopped by adding EDTA to 15 mM and SDS to 0.5%. Samples were then analyzed by electrophoresis through a 1.2% agarose gel as described in Appendix C. A, 30 mM Tris-HCl, pH 7.5, 2.5 ng topoisomerase; B, 30 mM Tris-HCl, pH 7.5, 12.5 ng topoisomerase; C, 30 mM Tris-HCl, pH 7.5, 2.5 ng topoisomerase, 200 ng acid-soluble protein; D, 30 mM sodium acetate, pH 6.0, 2.5 ng topoisomerase; E, 30 mM sodium acetate, pH 6.0, 12.5 ng topoisomerase; F, 30 mM sodium acetate, pH 6.0, 2.5 ng topoisomerase, 200 ng acid-soluble protein; G, 30 mM sodium acetate, pH 6.0, 200 ng acid-soluble protein; H, 30 mM sodium acetate, pH 6.0. Part B: Electrophoresis of the acid-soluble protein through a 15% SDS-polyacrylamide gel was carried out as described in Appendix E. A, molecular weight markers from top to bottom are: phosphorylase b (94,000), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibition (21,000); B, acid-soluble protein (25 µl).


A
B

A
B
Topo
pH 7.5
C
+ Acid soluble
fraction
D
E
Topo
pH 6.0
F
+ Acid soluble
fraction
G
H
Controls

Figure 13. Procedure for isolation of H1 histone from Ustilago.

All steps of the purification were carried out between 0-4°C. Cells freshly harvested (100 g) were washed 1 time in 200 ml 10 mM Tris, pH 7.5, 1 mM EDTA, 0.35 M NaCl. The cells were then resuspended in 300 ml of the same buffer and crushed at 20,000 psi by passage through a French pressure cell. The broken cells were then centrifuged in the JA 20 rotor of a Beckman J21-C centrifuge at 3500 rpm for 30 min. The supernatant was discarded and the pellet resuspended and washed 3 times in the above buffer. The pellet was then resuspended in 150 ml of 5% perchloric acid. After standing 10 min the suspension was centrifuged and the supernatant saved. This was repeated one more time and the pellet discarded. To the combined supernatants (≃ 300 ml) was then added 65 ml of 100% trichloroacetic acid (final concentration ≃ 18%). After standing for 10 min the resulting white precipitate was pelleted by centrifugation. The supernatant was discarded and the pellet washed 1 time with acid-acetone and 2 times with acetone. Residual acetone was removed by placing the pellet under vacuum. The yield of protein was approximately 1 mg.


Cells
Breakage in
0.35M salt
Supernatant
Sediment
5% Perchloric acid
extraction
Sediment
Supernatant
18% Trichloroacetic acid
Supernatant
Sediment
= 30K protein

to prevent proteolysis (77). However, since the yields of the H1-like protein did not appear to be dramatically improved, these reagents were subsequently left out. An average of 1 mg of pure protein could be obtained from 100 g of cells.

The purity and size of the acid-extracted H1-like protein was determined by electrophoresis through a SDS polyacrylamide gel (Fig. 14). By this criterion the Ustilago protein is greater than 95% pure and has a molecular weight of 30,000. Under these electrophoretic conditions it comigrates with calf thymus H1 histone. The two heavy staining bands for calf thymus H1 represent several different structural forms of H1 histone (57,58). The single staining band for the Ustilago H1-like protein suggests that lower eukaryotes lack some of these structural forms. Other alternatives are that structural forms of H1 were lost or degraded during the purification of the H1-like protein or that the gel system used was unable to resolve the different structural forms which might be present.

The ability of the H1-like protein from Ustilago to bind single-stranded DNA was tested and found to be strongly dependent upon pH (Fig. 15). Binding at pH 4 or 6 was half that observed at pH 5 where binding was found to be optimal. Essentially no binding of DNA occurred at pH 8.

One of the hallmarks of H1 histone is its special ability to bind superhelical DNA (55). The ability of the Ustilago protein to bind superhelical and relaxed forms of DNA was compared. As illustrated in Figure 16, binding of the H1-like protein to superhelical DNA was as much as 5-fold higher than to relaxed DNA which was in good agreement with the value reported for calf thymus H1 histone (78).

Figure 14. SDS polyacrylamide gel of H1 histone from Ustilago.

A 15% SDS polyacrylamide gel was prepared as described in Appendix E. A, Ustilago H1 histone (5 μg); B, calf thymus H1 histone (10 μg).

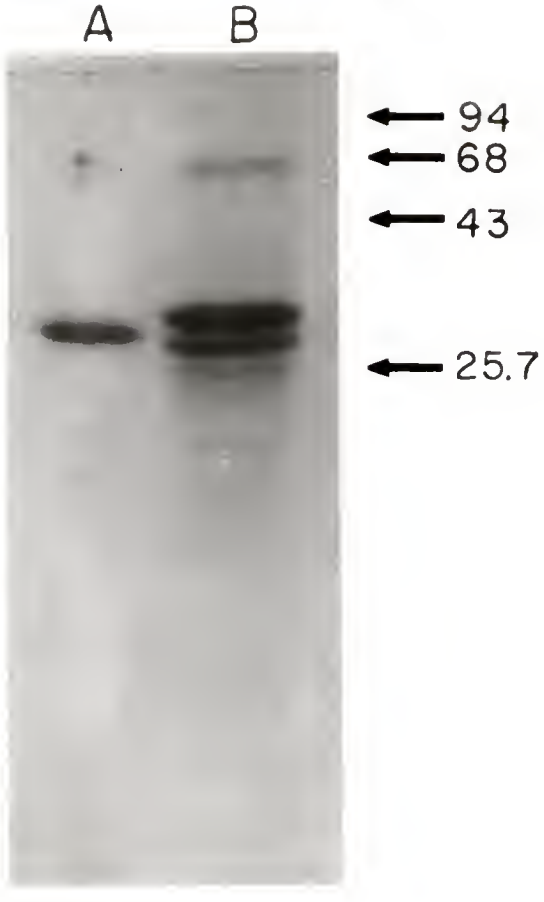

A
B
94
68
43
25.7

Figure 15. pH dependent binding of the Ustilago H1 to single-stranded DNA.

Reactions (50 µl) containing either 20 mM potassium phosphate (●) or sodium acetate (○) and 1 mM EDTA, 2 mM 2-mercaptoethanol, 5% glycerol, 50 mM NaCl, 1.5 nmol $\phi\chi$ phage [^{3}H] DNA and 1 µg of Ustilago H1 histone were incubated at 25°C. After 10 min reactions were terminated by adding 1 ml of the reaction buffer. The samples were then filtered through nitrocellulose filters and processed as described in Appendix C. Activity was determined relative to the value obtained at pH 5. At this pH approximately 80% of the input DNA was retained on the filter.

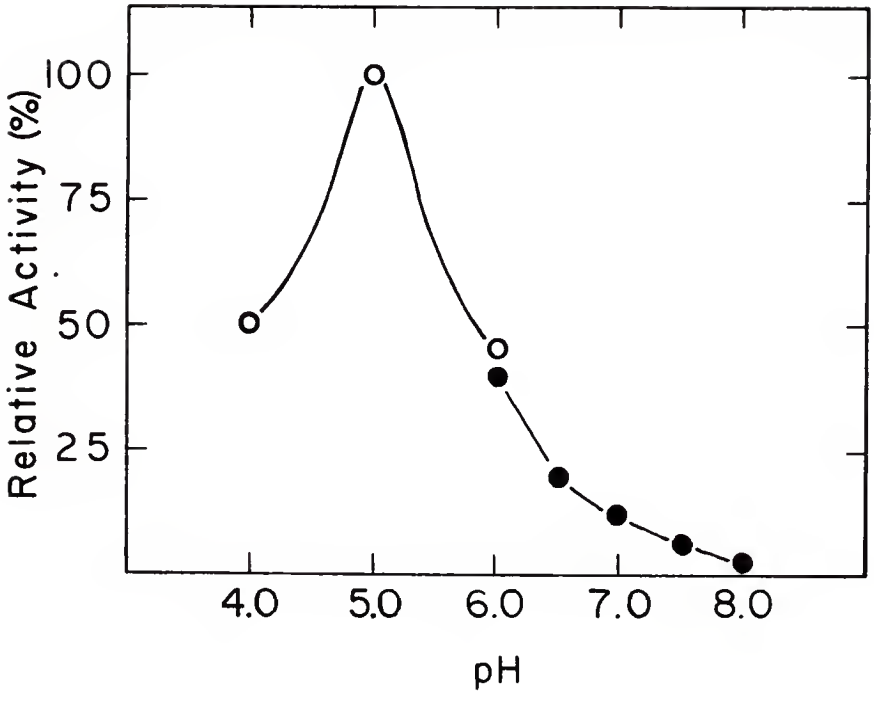
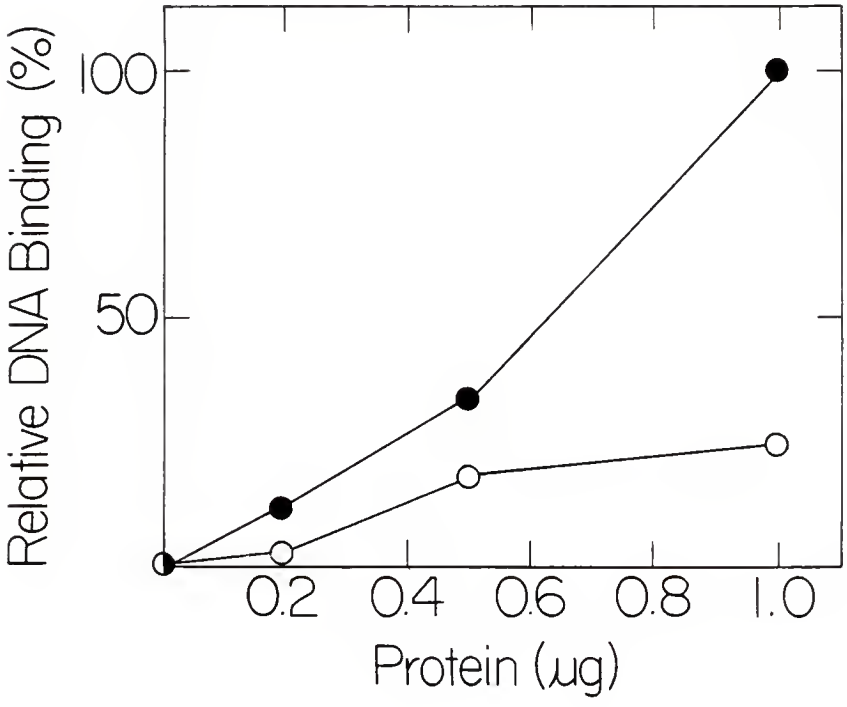

Relative Activity (%)
100
75
50
25
4.0
5.0
6.0
7.0
8.0
pH

Figure 16. Ability of the Ustilago H1 histone to bind superhelical DNA.

Reaction mixtures (50 µl) containing 50 mM sodium acetate, pH 6.0, 0.1 M, NaCl, 5 mM 2-mercaptoethanol, 5% glycerol and either fϑ RF I [^{3}H] DNA (●) or fϑ RF IV [^{3}H] DNA (○), were incubated with Ustilago H1 histone at 25°C. Reactions were stopped by the addition of 1 ml of reaction buffer and processed as described in Appendix C. Under these conditions, 1 µg of Ustilago H1 retained 0.1 nmol of fϑ RF I DNA to a nitrocellulose filter.


Relative DNA Binding (%)
100
50
0.2
0.4
0.6
0.8
1.0
Protein (μg)

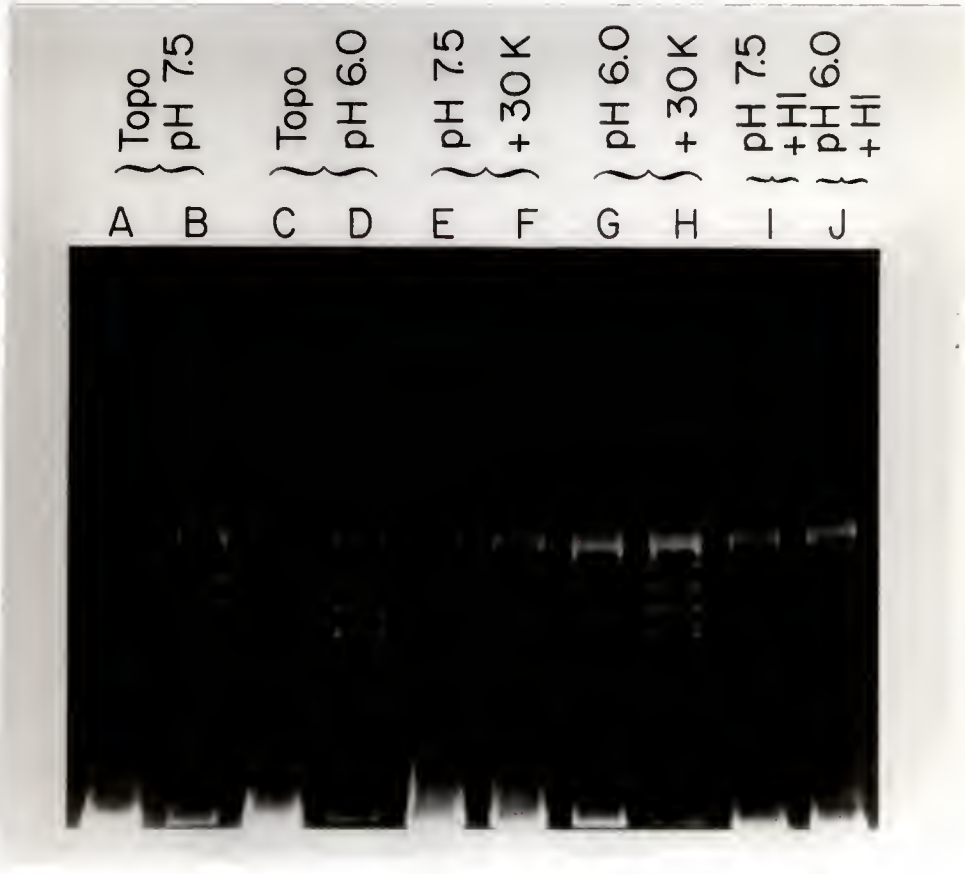
The H1-like protein stimulates topoisomerase activity! However, stimulation is pH-dependent (Fig. 17). At pH 7.5, where the H1-like protein exhibits little binding to DNA, only slight stimulation of topoisomerase activity is observed. However, at pH 6, where its binding to DNA is high, the H1-like protein stimulated topoisomerase activity greater than 5-fold. Although the stimulatory protein displayed no relaxation activity in the absence of added topoisomerase, low levels of endonuclease activity were detectable at pH 6. The ratio of Ustilago H1-like protein to DNA required to obtain significant stimulation of the topoisomerase was 0.25 (w/w). This is 5-fold higher than the ratio of calf thymus H1 to DNA necessary to induce a similar level of stimulation at pH 6. Unlike the Ustilago protein, calf thymus H1 effectively binds to DNA over the entire pH range from 6 - 8 (unpublished results, 78). It is also able to stimulate the Ustilago topoisomerase at both pH 6 and 7.5. This suggests that stimulation of the topoisomerase by either calf thymus H1 or the Ustilago H1-like protein requires their ability to bind DNA.

A 25,000 Dalton Acid-Soluble Protein also Stimulates Topoisomerase Activity

Another acid-soluble protein has been isolated from Ustilago which also is able to stimulate topoisomerase activity. This protein was purified from the 0.35 M salt supernatant normally discarded during the purification of the H1-like protein (refer to Fig. 2). Extraction of the 0.35 M salt supernatant with acid and subsequent chromatography of the acid-soluble fraction on carboxymethylcellulose yielded a homogenous preparation of this protein (refer to Appendix D for details of the

Figure 17. Stimulation of topoisomerase activity by Ustilago H1 histone.

Reactions (25 µl) containing either 25 mM Tris-HCl, pH 7.5 (lanes A, B, E, F, I) or 25 mM sodium acetate, pH 6.0 (lanes C, D, G, H, J), and 10 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 5% glycerol, 10 µg/ml BSA and the following additions were incubated at 25°C. After 30 min the reactions were stopped by adding EDTA to 15 mM and SDS to 0.5%. The products of the reactions were then analyzed by electrophoresis through a 1.2% agarose gel as described in Appendix C. A and C, 2 ng topoisomerase; B and D, 10 ng topoisomerase; E, 2 ng topoisomerase, 0.25 µg Ustilago H1 histone; F, 2 ng topoisomerase, 1.0 µg Ustilago H1 histone; G, 2ng topoisomerase, 0.25 µg Ustilago H1 histone; H, 2 ng topoisomerase, 1.0 µg Ustilago H1 histone; I and J, 2 ng topoisomerase, 50 ng calf thymus H1 histone.


Topo
pH 7.5
Topo
pH 6.0
pH 7.5
+ 30 K
pH 6.0
+ 30 K
pH 7.5
+ HI
pH 6.0
+ HI
A
B
C
D
E
F
G
H
I
J

purification). As determined from electrophoresis through a 15% SDS polyacrylamide gel, it has a molecular weight of 25,000. This is very similar to the molecular weight of one of the Ustilago DNA binding proteins, DBP IV (refer to Appendix D). A subsequent comparison of the electrophoretic mobilities of DBP IV and the 25,000 dalton acid-soluble protein showed them to be nearly identical (Fig. 18). If DBP IV is structurally related to the 25,000 dalton protein it should be soluble in acid. To test this, DBP IV was extracted with 5% perchloric acid and the acid-soluble and acid-insoluble fractions subsequently electrophoresed through a SDS polyacrylamide gel. As is evident from Figure 19, DBP IV appears only in the acid-soluble fraction.

DBP IV strongly stimulated the Ustilago topoisomerase. Similar to calf thymus H1, but unlike the Ustilago H1-like protein, DBP IV stimulated topoisomerase activity at both pH 6 and 7.5 (Fig. 20). DBP IV also displayed an ability to bind to DNA equally well at both of these pHs (Fig. 21). At a protein to DNA ratio of 0.05 (w/w) DBP IV was equally as effective as calf thymus H1 histone in stimulating topoisomerase activity.

Conclusions

An H1-like protein purified from Ustilago stimulates relaxation of superhelical DNA by the topoisomerase. On SDS polyacrylamide gels it comigrates with calf thymus histone H1. Like calf thymus H1 the Ustilago protein is soluble in 5% perchloric acid and shows a preferential binding to superhelical DNA (55). In contrast to calf thymus H1, the Ustilago H1-like protein binds DNA in a pH-dependent fashion (78). The ability of the H1-like protein to stimulate topoisomerase activity

Figure 18. Electrophoresis of a 25,000 dalton acid-soluble protein and DBP IV on a SDS polyacrylamide gel.

A 15% SDS polyacrylamide gel was prepared and run as described in Appendix E. Protein was visualized using the silver staining technique. Molecular weight markers included BSA, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor. A, DBP IV (1.0 µg); B, acid-soluble protein (3 µg).

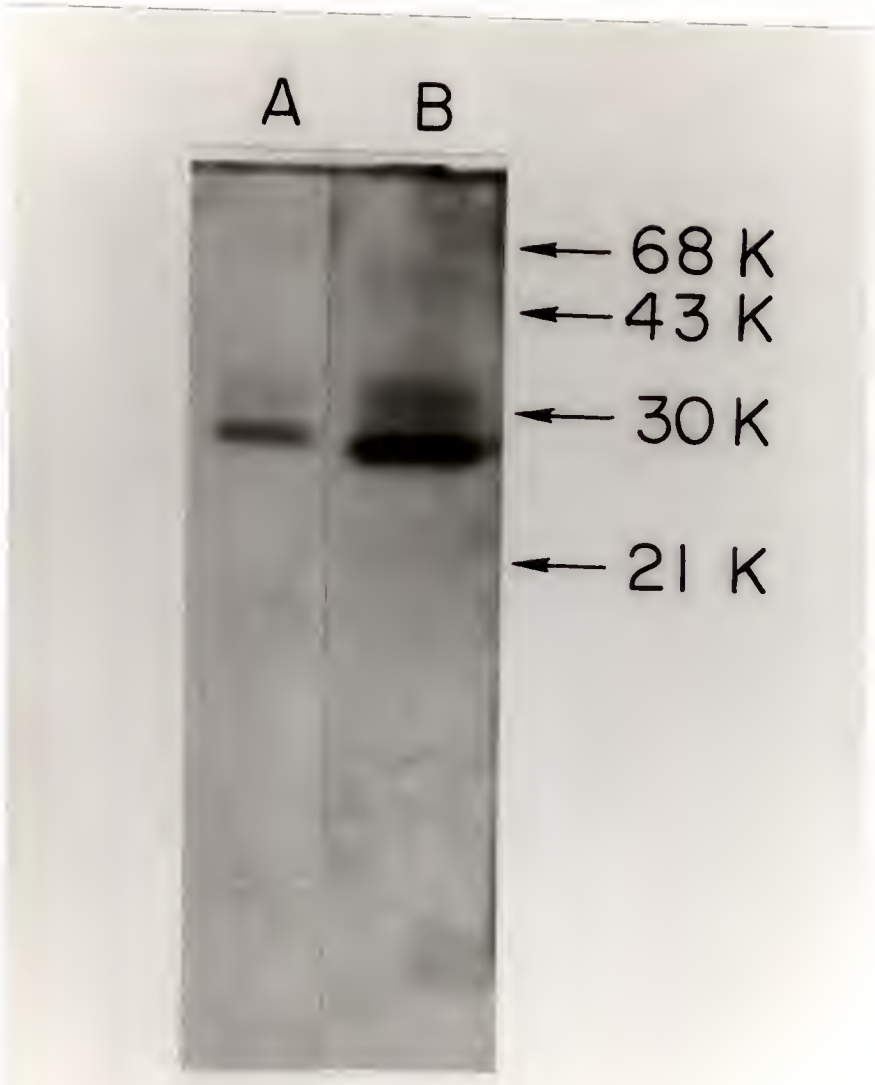

A
B
68 K
43 K
30 K
21 K

Figure 19. Acid-solubility of DBP IV.

Approximately 5 μg of DBP IV from fraction II of the purification was extracted with perchloric acid and analyzed as described in the legend to Figure 12. Protein was visualized using the silver staining technique as described in Appendix E. Molecular weight markers included ovalbumin, α-chymotrypsinogen and β-lactoglobulin. A, Perchloric acid precipitate; B, Perchloric acid supernatant.

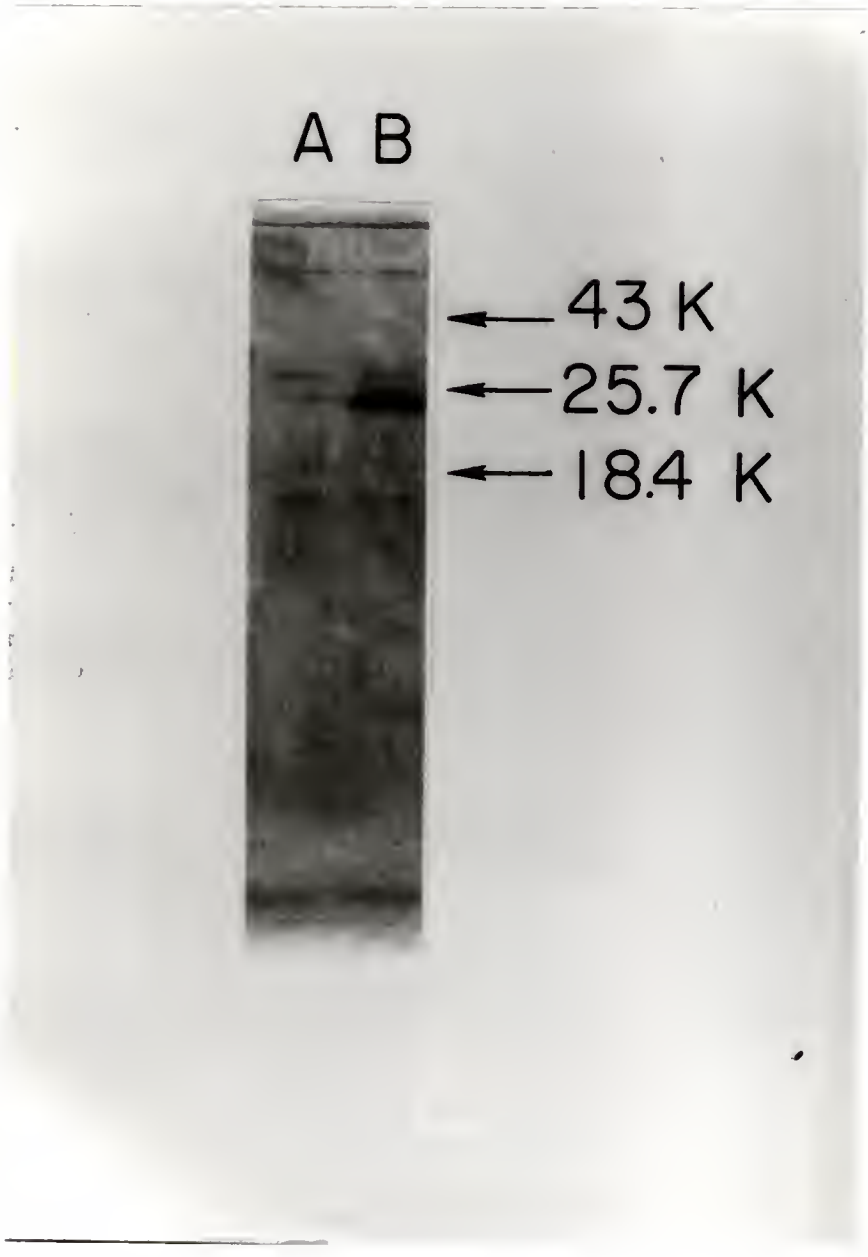

A B
43 K
25.7 K
18.4 K

Figure 20. Stimulation of the topoisomerase by DBP IV.

Reactions (25 μl) containing 25 mM Tris-HCl, pH 7.5 (lanes A, B, C, G, H) or 25 mM sodium acetate, pH 6.0 (lanes D, E, F, I), and 10 mM $MgCl_2$, 0.4 mM 2-mercaptoethanol, 5% glycerol, 10 μg/ml BSA and the following additions were incubated at 25°C. After 30 min the reactions were terminated and the products analyzed as described in the legend to Figure 17. A and D, 2.5 ng topoisomerase; B and E, 12.5 ng topoisomerase; C and F, 2.5 ng topoisomerase, 100 ng DBP IV; G and H, 100 ng DBP IV; I, no additions.

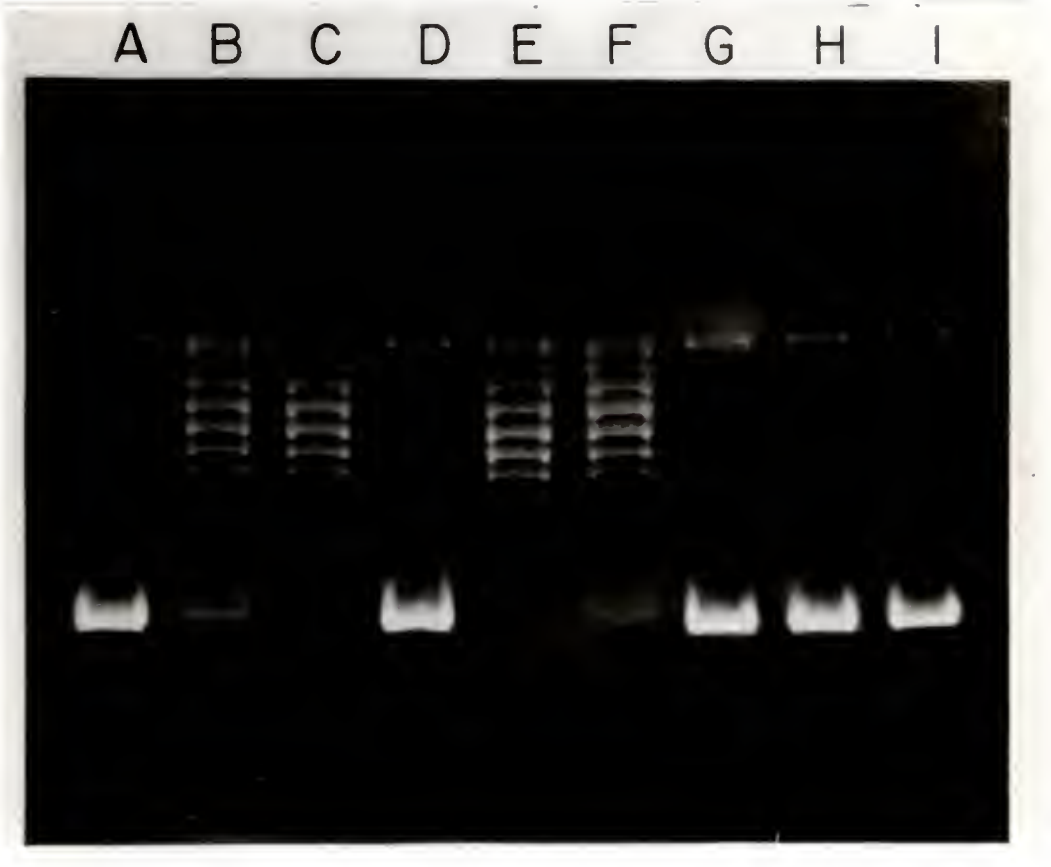
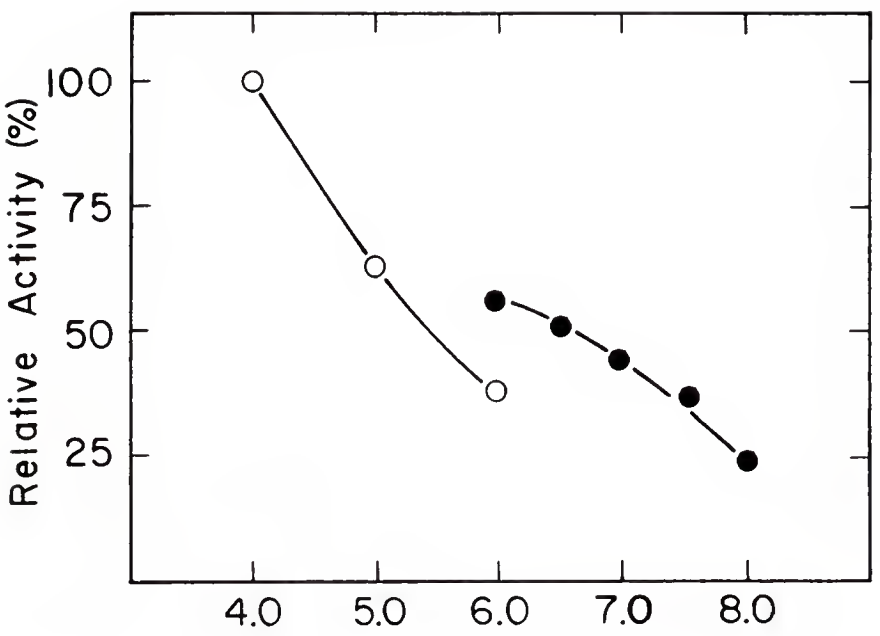

A
B
C
D
E
F
G
H
I

Figure 21. Effect of pH on the ability of DBP IV to bind single-stranded DNA.

Reaction (50 µl) containing either 20 mM potassium phosphate (●) or sodium acetate (○) and 1 mM EDTA, 2 mM 2-mercaptoethanol, 5% glycerol, 50 mM NaCl, 1.5 nmol $\phi\chi$phage [^{3}H] DNA (2x 10^7 cpm/µmol) and 250 ng DBP IV were incubated at 25°C. After 10 min, the reactions were terminated and analyzed as was described in the legend to Figure 15. Activity was determined relative to the value obtained at pH 7.5. At this pH, DBP IV caused the retention of approximately 92% of the DNA to the nitrocellulose filter.


Relative Activity (%)
100
75
50
25
4.0
5.0
6.0
7.0
8.0

is also pH dependent and directly correlates with its ability to bind DNA. This suggests an interaction between the Ustilago H1-like protein and DNA is necessary for stimulation of the topoisomerase.

H1-like proteins have been isolated from several other fungi and the amino acid contents of these proteins are known (72-76). Therefore, an amino acid analysis of the Ustilago H1-like protein should provide important information concerning its structure and identity.

During the course of purifying the H1-like protein, a second acid-soluble protein was discovered which also showed an ability to stimulate topoisomerase activity. This 25,000 molecular weight protein was present in the 0.35 M salt supernatant which was normally discarded during the purification of the H1-like protein. The purification scheme for the 25,000 dalton protein was very similar to a procedure used by Johns to isolate high-mobility group (HMG) proteins (79-81). HMGs are highly charged, low molecular weight proteins which are thought to play an important role in controlling DNA structure (82-84). Additional studies have been undertaken to determine if the 25,000 dalton Ustilago protein is an HMG. These studies have been encouraged by recent work in another laboratory which showed that HMG 17 strongly stimulated relaxation of superhelical DNA by a type I topoisomerase from HeLa cells. There has also been a report that topoisomerase activity has been found in association with HMG 14 and HMG 17 in actively transcribing regions of chromatin (84).

The 25,000 dalton protein may be structurally related to a Ustilago DNA binding protein, DBP IV. Their molecular weights were found to be identical when determined by electrophoresis through SDS

polyacrylamide gels. Like the 25,000 dalton protein, DBP IV was soluble in 5% perchloric acid. In addition, DBP IV showed a similar ability to stimulate the Ustilago topoisomerase. Studies aimed at elucidating the primary structure of these two proteins are in progress and should reveal if they are indeed related.

CHAPTER IV
CHARACTERIZATION OF THE COVALENT TOPOISOMERASE-DNA LINKAGE

One of the trademarks of topoisomerases is their ability to form a transient covalent complex with DNA (1-3). Breakage of DNA by prokaryotic topoisomerases and eukaryotic type II topoisomerases results in the formation of a covalent complex between the enzyme and the 5' end of the DNA break (6,12). Eukaryotic type I topoisomerases, on the other hand, form a covalent complex with the 3' end of the DNA break (13,14). Both eukaryotic and prokaryotic enzymes are linked to the DNA through a phosphotyrosine bond (9,10). The energy conserved in the phosphotyrosine linkage is thought to drive the reclosing of the transient break in the DNA.

Although the mechanism of action for topoisomerases isolated from prokaryotic as well as higher eukaryotic organisms has been well studied, no such studies have been undertaken in lower eukaryotic organisms. This is unfortunate since lower eukaryotic organisms provide such an ideal genetic setting for testing the relevance of biochemical studies. The following work represents an initial effort to bridge this present gap in biochemical knowledge.

Detection of the Covalent Complex

Under conditions of low salt (<50 mM), rat liver type I topoisomerase breaks circular single-stranded phage DNA to form a linear DNA molecule with enzyme covalently attached at the 3' end. Subsequent incubation of the DNA-protein complex in the presence of 0.25 M salt or 10 mM $MgCl_2$ leads to the recircularization of the DNA (39).

The Ustilago topoisomerase also breaks circular single-stranded phage DNA to form linear DNA molecules with protein tightly attached. Conversion of circular single-stranded DNA to a linear form can be easily assessed by electrophoresis of the DNA through agarose gels containing 30 mM NaOH. Topoisomerase was incubated with circular single-stranded fϑ DNA at pH 9.0 in reactions containing low concentrations of salt (<50 mM). Analysis of the products of this reaction on an alkaline agarose gel revealed a significant decrease in the amount of circular DNA present with a corresponding increase in a DNA species which migrated slightly slower than unit-length linear single-stranded fϑ DNA (Fig. 22 A). Treatment of the reaction products with 0.5% SDS prior to electrophoresis did not alter the gel pattern (data not shown). However, if the reaction products were treated with proteinase K prior to electrophoresis, the DNA band present in lane A vanished and a new band appeared having an electrophoretic mobility identical to linear single-stranded fϑ DNA (Fig. 22 B).

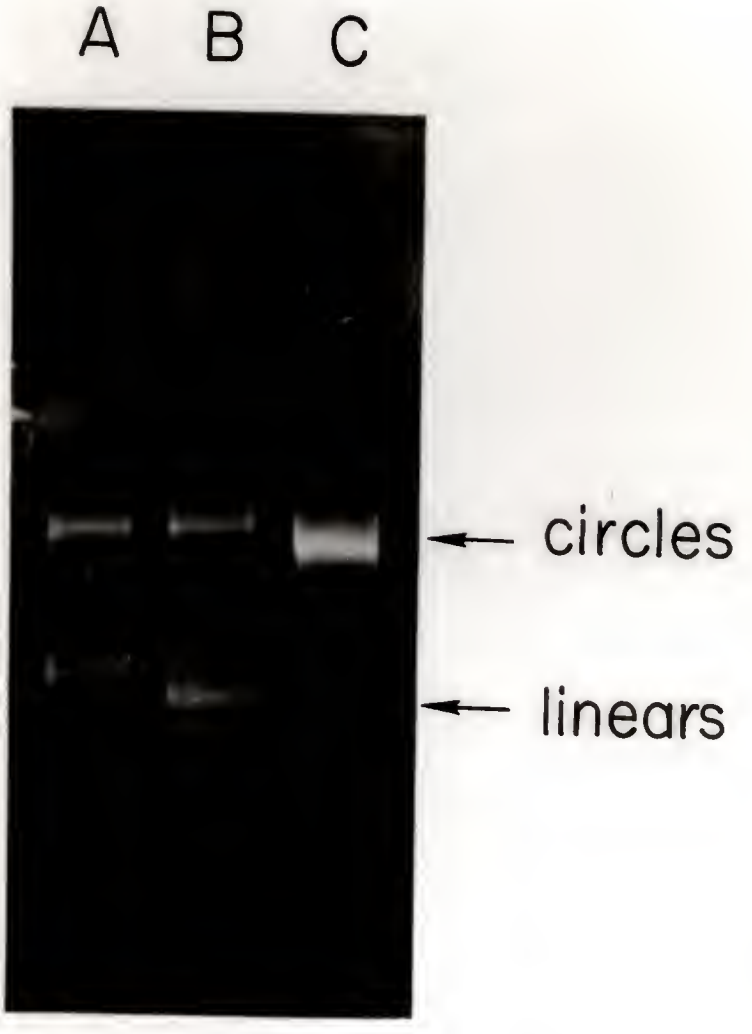
Attempts to recircularize the fϑ DNA which had been broken by the Ustilago topoisomerase were unsuccessful. Reactions containing circular single-stranded fϑ DNA previously broken by the topoisomerase were incubated in the presence of 0.2 M salt or 10 mM $MgCl_2$ for 2 hr at 25°C. Subsequent electrophoresis of the reactions on alkaline agarose gels revealed no significant recircularization of the DNA (data not shown).

Identification of the Protein Complex to the DNA

The inability to disrupt the complex formed between the Ustilago topoisomerase and single-stranded DNA, by treatment with 30 mM NaOH or

Figure 22. Covalent attachment of the topoisomerase to DNA.

Reactions (40 µl) containing 10 mM Tris-HCl, pH 9.0, 1 mM EDTA, 0.2 mM dithiothreitol, 50 µg/ml BSA, 5% glycerol, 2.4 nmol fə phage DNA, and either 1 µg of topoisomerase (lanes A and B) or no topoisomerase (lane C) were incubated at 25°C for 20 min. Reactions A and C were terminated by adding 4 µl of 0.3 M NaOH. Reaction B was treated with proteinase K (100 µg/ml) for 30 min at 37°C before adding 4 µl of 0.3 M NaOH. Fifteen µl of each reaction was loaded onto a 1.2% agarose gel containing 30 mM NaOH, 0.5 mM EDTA. Electrophoresis was at 5.5 v/cm for 16 hr at 5°C. Following electrophoresis the gel was stained with 1 µg/ml eithidium bromide before photographing under ultraviolet light.

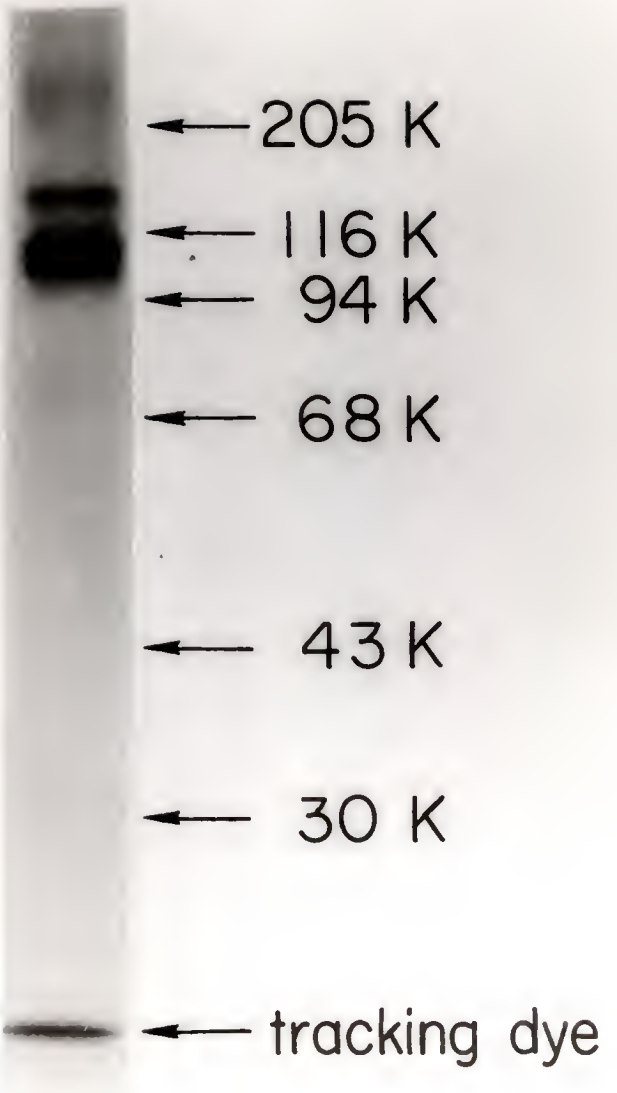

A
B
C
circles
linears

0.5% SDS suggested that a covalent protein-DNA linkage was involved. To determine the identity of the protein linked to the DNA, the Ustilago topoisomerase was incubated with denatured $\phi\chi174$ DNA which had been labeled by nick translation with [α^{32}P] dCTP. The resulting DNA-protein complex was digested with Micrococcal nuclease and then extracted with phenol. The protein in the phenol phase was recovered by acetone precipitation and electrophoresed through an SDS polyacrylamide gel. The gel was then dried down on Whatman 3 MM paper and autoradiographed. The three radiolabeled protein bands present in Figure 23 have molecular weights of 100,000, 110,000 and 130,000. When the radiolabeled protein was treated with proteinase K prior to electrophoresis, no radioactive bands were detectable on the autoradiograph (data not shown) confirming that the bands present in Figure 23 represented proteins tightly coupled to short pieces of DNA.

Eukaryotic type I topoisomerases are highly susceptible to proteolytic degradation during enzyme purification and this may explain why there is more than one radiolabeled protein present in the autoradiograph in Figure 23. Eukaryotic type I topoisomerases isolated from HeLa cells and Drosophila melanogaster, under conditions which minimized proteolytic degradation, yielded single polypeptides with molecular weights of 120,000 and 110,000 respectively (2). Perhaps greater precautions against proteolytic degradation during the purification of the Ustilago topoisomerase will yield a single polypeptide.

Figure 23. Analysis of the enzyme-oligonucleotide complex by SDS gel electrophoresis.

The procedures used to label and denature the DNA are described in Appndix B. A 150 µl reaction containing 6 mM Tris-HCl, pH 9.0, 0.2 mM dithiothreitol, 50 µg/ml BSA, 150 pmol denatured nick translated [^{3}H] PBR DNA (3.3 x 10^7 cpm/nmol) and 1 µg topoisomerase was incubated at 25°C for 20 min. The reaction was stopped by adding NaOH to 50 mM and incubating for 5 min at 37°C. The reaction was then made 30 mM in Tris-HCl and the pH titrated to 9.0 with HCl. After adding $CaCl_2$ to a final concentration of 2 mM, 0.1-0.2 units of Micrococcal nuclease were added. After incubating at 37°C for 1 hr the reaction was extracted with 1 volume of phenol. The phenol phase was saved and extracted 6 times with equal volumes of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. The protein-oligonucleotide complex was precipitated from the phenol phase by adding 10 volumes ice-cold acetone and placing the sample in a dry ice-ethanol bath. After 20 min the sample was centrifuged and the resulting pellet resuspended in sample buffer. After heating at 100°C for 3 min, the sample was analyzed by electrophoresis through a 7.5% SDS polyacrylamide gel as described in Appendix E. Following electrophoresis the gel was stained with Coomassie blue to visualize the marker protein. The gel was then dried down on Whatman 3 mm paper and autoradiographed using Kodak X-Omat AR 5 film with a Dupont lightning-plus intensifying screen at -70°C.


205 K
116 K
94 K
68 K
43 K
30 K
tracking dye

Topoisomerase Links to the 3' End of the DNA Break

Two approaches were taken to determine whether the Ustilago topoisomerase was linked to the 3' or 5' end of the broken strand of DNA. In the first approach [^{3}H] labeled circular single-stranded fϑ DNA, which had been broken by the topoisomerase, was treated with exonuclease I or exonuclease VII. If breakage of the DNA by the topoisomerase produces a free 3' hydroxyl terminus the broken DNA should be susceptible to digestion by both exonuclease I and exonuclease VII. However, if breakage produces a free 5' hydroxyl terminus the broken fragment of DNA should only be susceptible to digestion by exonuclease VII. The results of this experiment are presented in Table V and show that breakage of the DNA by the Ustilago topoisomerase produces a free 5' terminus suggesting that the topoisomerase is linked to the 3' side of the DNA break. This result also points out that cleavage of DNA by the topoisomerase occurs under normal reaction conditions and is not simply an artifact of stopping the reaction with alkali or detergent.

To determine whether the topoisomerase was linked to DNA at the 3' or 5' end, complexes formed between fϑ phage [^{3}H] DNA (2×10^4 cpm/nmol) and topoisomerase were treated with exonuclease I (exo I) or exonuclease VII (exo VII). A 75 µl reaction containing 10 mM Tris-HCl, pH 9.0, 0.2 mM dithiothreitol, 50 µg/ml BSA, 5 nmol fϑ phage [^{3}H] DNA and 1 µg topoisomerase was incubated at 25°C. After 10 min three 20 µl aliquots were removed and analyzed as described in Appendix C. One aliquot was diluted into a 200 µl reaction containing 0.8 units of exonuclease I. Another aliquot was diluted into a 200 µl reaction containing 0.6 units of exonuclease VII. The third aliquot was electrophoresed through a

TABLE V
DIGESTION OF TOPOISOMERASE-DNA COMPLEXES WITH EXONUCLEASE I AND EXONUCLEASE VII

	Acid Solubility Percent		Circular DNA Percent
	Exo I	Exo VII	
Control	5	9	100
Topoisomerase	6	58	41

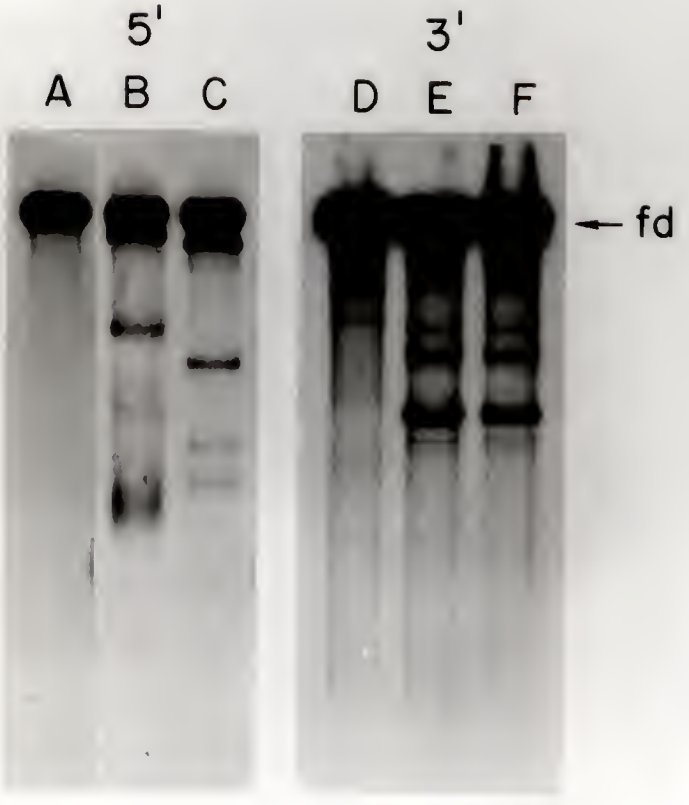
1.2% alkaline agarose gel as described in the legend to Figure 22. A control reaction containing no topoisomerase was processed in the same manner. The percent circular DNA present was determined from densitometer tracings of the photographic negative of the agarose gel.

The attachment of the topoisomerase to the 3' end of a broken DNA strand was shown in another way. Form III fϑ DNA, produced by digesting fϑ RFI DNA with the restriction endonuclease Sau 96, was labeled with [γ-^{32}P] ATP at its 5' ends using T4 polynucleotide kinase. The DNA was denatured and incubated with the Ustilago topoisomerase. Electrophoresis of the reaction products on an alkaline agarose gel revealed several major bands (Fig. 24 B). If the reaction products were treated with proteinase K prior to electrophoresing the mobilities of these bands were shifted (Fig. 24 C). This is what would be expected if the topoisomerase was linked to the 3' end of the broken DNA strand. When the same experiment was carried out with DNA labeled with [γ-^{32}P] dCTP at its 3' ends, using the large Klenow fragment of E. coli DNA polymerase, no shift in the mobility of the reaction products was observed following treatment with proteinase K (Figs. 24 E and F). The results from this study in combination with the exonuclease digestion results provide strong evidence for the tight attachment of the Ustilago topoisomerase to the 3' and of the broken DNA strand.

Breakage of end-labeled DNA by the topoisomerase produces discrete bands (Figs. 24 B, C, E, F) inferring that cleavage of the DNA is site-specific. Type I topoisomerases from HeLa cells, calf thymus and rat liver also cleave DNA in a site-specific fashion (85,86). As determined from DNA sequencing, the cut sites on SV40 DNA for the HeLa cell and calf

Figure 24. Proteinase K treatment of topoisomerase complexed with DNA labeled at the 3' or 5' end.

Form III fϑ DNA, prepared by digesting form I fϑ DNA with the restriction endonuclease Sau 96, was labelled with [^{32}P] at the 3' or 5' end as described in Appendix B. Denaturation of the DNA prior to reacting with the topoisomerase was carried out as described in Appendix B. Reactions (40 µl) containing 10 mM Tris-HCl pH 9.0, 0.2 mM dithiothreitol, 50 µg/ml BSA, and 0.3 nmol of denatured 5' (lanes A-C) or 3' (lanes D-F) [^{32}P] end labeled DNA were incubated with (lanes B, C, E, F) or without (lanes A and D) 1 µg of topoisomerase at 25°C for 30 min. Reactions were terminated by adding 4 µl of 0.3 M NaOH. Prior to adding NaOH, samples in lanes C and F were treated with proteinase K (100 µg/ml) for 30 min at 37°C. Twenty µl of each sample was analyzed by electrophoresis through a 1.6% agarose gel containing 30 mM NaOH, 1 mM EDTA at 10 v/cm for 5 hr at 5°C. Following electrophoresis the gel was soaked for 10 min in 500 ml of 0.1 M Tris-HCl pH 7.5 before it was dried down on Whatman 3 mm paper. Autoradiography was carried out as described in the legend to Figure 23.


5'
3'
A
B
C
D
E
F
fd

thymus topoisomerases are identical. However, there does not appear to be a consensus sequence for determining cleavage (87). It has been suggested that secondary structure may be a major factor contributing to the site-specific cleavage of DNA by type I topoisomerase (86).

Identification of the Covalent Linkage

Both eukaryotic and prokaryotic topoisomerases form covalent complexes with DNA involving a phosphotyrosine bond (9,10). To determine if the Ustilago topoisomerase was covalently linked to DNA through a phosphotyrosine bond, enzyme was complexed to denatured ϕX174 RF DNA which had been labeled by nick translation with [γ-^{32}P] dCTP. Following digestion with Micrococcal nuclease, the complexes were purified by phenol extraction and acetone precipitation as described in the legend to Figure 23. The purified protein-oligonucleotide complexes were then hydrolyzed in 5.6 M HCl at 110°C for 2 hr and the products of hydrolysis analyzed by high voltage paper electrophoresis (Fig. 25). Under these conditions of hydrolysis both DNA and protein should be broken down to produce inorganic phosphate and amino acids respectively. However, a phosphotyrosine bond, if present, should be stable. Almost all of the radioactivity present in the autoradiograph in Figure 25 is contained in two spots. The upper spot comigrated with inorganic phosphate while the lower remaining spot of radioactivity comigrated with the phosphotyrosine marker suggesting that the covalent linkage between the Ustilago topoisomerase and DNA involves a phosphotyrosine bond.

Discussion

In summary, the Ustilago topoisomerase breaks single-stranded DNA in low salt forming a tight complex with the 3' side of the DNA break.

Figure 25. Paper electrophoresis of the [^{32}P] labeled topoisomerase-oligonucleotide complex following acid hydrolysis.

The topoisomerase-oligonucleotide complex was obtained as described in the legend to Figure 23. The complex which had been precipitated from the phenol phase was resuspended in 40 µl of 5.6 M constant boiling HCl. The sample was sealed in a 1.4 ml Eppendorf tube under a nitrogen atmosphere before heating in a 110°C oil bath. After 2 hr the sample was cooled and the HCl removed *in vacuo* in the presence of NaOH pellets. The resulting residue was resuspended in 30 µl of H_2O containing 100 nmol each of phosphotyrosine, Tyr(P), phosphoserine, Ser(P), and phosphothreonine, Thr(P), and 500 nmol of sodium phosphate pH 7.0, P_i. The sample was analyzed by paper ionophoresis as described in Appendix E. Autoradiography was carried out as described in the legend to Figure 23. The dashed lines represent the locations of the marker compounds which were visualized as described in Appendix E.

P_i
Ser(P)
Thr(P)
Tyr(P)

This complex probably involves a covalent linkage since neither alkali or detergent were able to disrupt it. In addition, acid hydrolysis of the radiolabeled complex yielded a radioactive product which coelectrophoresed with authentic phosphotyrosine implying that the linkage was not only covalent, but that it involved a phosphotyrosine bond.

The Ustilago enzyme, in contrast to the rat liver type I topoisomerase, appears to be unable to reseal the break which it introduces into single-stranded DNA. Circular single-stranded fϑ DNA broken by the Ustilago topoisomerase does not recircularize in the presence of either 0.2 M salt or 10 mM $MgCl_2$, conditions which are normally optimal for the nicking-closing activity of this enzyme on duplex superhelical DNA. Perhaps the problem of reclosure is not an enzymatic one but reflects the low frequency with which the two ends of the broken strand come into close enough alignment for religation to occur. This would not be a problem in a duplex DNA molecule where the stable secondary structure of the DNA would be expected to maintain the ends of a break in close alignment ensuring efficient reclosure of the transient break created by the topoisomerase.

Topoisomerases join a host of other proteins in being able to form a covalent protein-nucleic acid linkage. The poliovirus encoded protein VPg forms a covalent linkage with the 5' end of poliovirus RNA. Like topoisomerases, this covalent linkage involves a phosphotyrosine bond (87,88). Covalent protein-nucleic acid complexes have also been described for a number of other viruses (89-91). These covalent complexes are thought to play an important role in the initiation of replication. Whether topoisomerases can function in a similar manner is at

present unknown. The covalent attachment of eukaryotic type I topoisomerases to the 3' rather than the 5' end of the DNA suggests they are probably not involved in the priming of DNA replication. However, this does not preclude eukaryotic type I topoisomerases from playing a role in removing any topological constraints which might arise during the course of DNA replication.

CHAPTER V
INVOLVEMENT OF THE *Ustilago* TOPOISOMERASE IN GENETIC RECOMBINATION

The biological role of topoisomerases in prokaryotic genetic recombination has recently been established. Mutations in the gene coding for *E. coli* DNA topoisomerase I (*omega* protein) have been found to dramatically reduce the transposition frequency of the antibiotic-resistance-carrying transposons Tn 5, Tn 9 and Tn 10 (37). Studies on another topoisomerase in *E. coli*, DNA gyrase, have provided evidence for a role of this enzyme in prokaryotic illegitimate and site-specific recombination (26,38).

The role of topoisomerases in eukaryotic genetic recombination is less clear. Although *in vitro* studies on several eukarytoic type I topoisomerases suggest a possible involvement of these enzymes in both homologous and illegitimate forms of recombination, there is no genetic evidence to support a similar involvement *in vivo* (3).

Recently, biochemical as well as genetic evidence have been obtained which suggest an involvement of the *Ustilago* topoisomerase in DNA recombination. The evidence and its possible significance are discussed in this chapter.

The *Ustilago* Topoisomerase Recombines Complementary Single-Stranded DNA Rings

In the past it was believed that homologous recombination required DNA with free ends. However, with the discovery that type I topoisomerases could catalyze recombination between complementary single-stranded

DNA circles to form closed circular duplex DNA, a new alternative to strand transfer was established bypassing the need for free ends in the DNA (22,23).

The Ustilago topoisomerase also appears to catalyze intertwining of complementary single-stranded circles of DNA. To illustrate this property, complementary single-stranded circles of ϕX174 [^{3}H] DNA were incubated with the Ustilago topoisomerase under the reaction conditions described in the legend to Figure 26. The products of the reaction were then centrifuged through an alkaline sucrose gradient. As is evident from Figure 26 A, treatment of complementary single-stranded circles of ϕX174 DNA with the topoisomerase leads to the formation of a DNA species which sediments at a rate similar to denatured covalently closed duplex ϕX174 DNA suggesting that the topoisomerase is able to topologically intertwine complementary circles of DNA. If the topoisomerase is incubated with ϕX174 phage DNA, which consists of only "plus" strands, no fast sedimenting DNA species is observed suggesting this reaction requires that the DNA circles be complementary (data not shown).

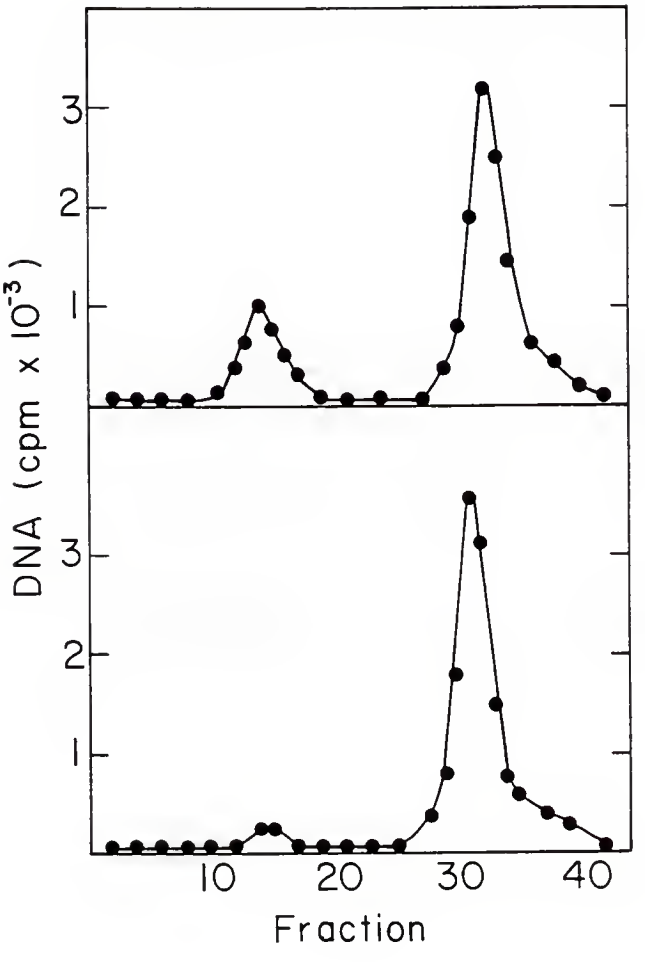
The ability of type I topoisomerases to catalyze intertwining of complementary circular strands of DNA suggests an in vivo role for these enzymes in removing topological barriers which might arise during DNA recombination. Such barriers could easily be envisioned to occur during strand exchange between two DNA molecules.

Relaxing Activity Reduced in the rec 2 Mutant

Mutants of Ustilago defective in DNA replication, repair, and recombination were screened for the presence of topoisomerase activity using the agarose gel assay. A mutant defective in recombination, rec 2,

Figure 26. Interlocking of complementary single-stranded rings of DNA by the topoisomerase.

Reactions (50 µl) containing 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 25 mM KCl, 5% glycerol, 2 nmol complementary single-stranded $\phi\chi[^3H]$ DNA rings, and (A) 200 ng of topoisomerase or (B) no topoisomerase were incubated at 30°C. After 20 min the reactions were terminated by adding 50 µl or 1 M NaCl, 10 µl 0.25 M EDTA and 100 µl 0.5 M NaOH. Samples were then loaded onto 5-20% sucrose gradients containing 0.25 M NaOH 1 mM EDTA, and 0.75 M NaCl. The gradients were centrifuged at 40,000 rpm in the SW 40.1 rotor of a Beckman L5-50 centrifuge at 4°C for 4 hr. Forty-two fractions (0.25 ml each) were collected from the bottom of each gradient and each fraction was neutralized with 0.25 ml of 0.25 M HCl before adding 5 ml of Formula 950 scintillation fluor (New England Nuclear). The radioactivity of each sample was then determined. Purification of the $[^3H]$ $\phi\chi$ complementary single-stranded rings of DNA used in the above reactions is described in Appendix B.


DNA (cpm x 10^{-3})
3
2
1
3
2
1
10
20
30
40
Fraction

showed a significantly reduced level of DNA relaxing activity. As shown in Figure 27, an extract prepared from rec 2 cells contained less than 20% of the relaxing activity of extracts from rec + cells.

The heat stabilities of enzyme partially purified from rec 2 and rec + cells were also compared and suggests that the topoisomerase from the mutant may be structurally altered (Dr. M. Brougham, personal communication). However, these results are preliminary in nature and additional studies will be needed to determine if the rec 2 mutant contains a structural defect in the gene coding for the Ustilago topoisomerase.

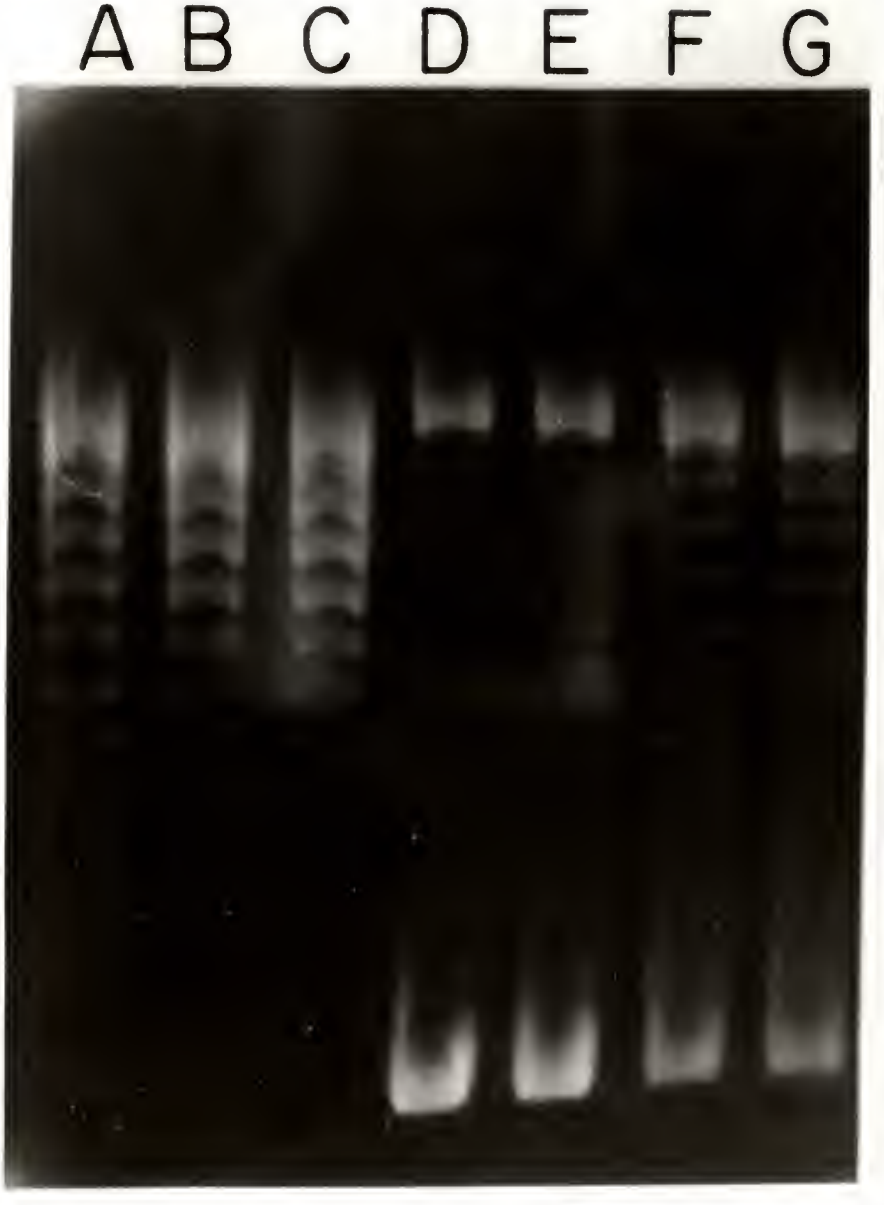
Discussion

The Ustilago topoisomerase can carry out several biochemical reactions which may be of consequence in genetic recombination. One of these reactions, the topological isomerization of DNA, may determine the effectiveness of DNA as a substrate in recombination. The role of superhelicity in bacteriophage lambda as well as transposon $\gamma\delta$ recombination points out how the topological state of DNA can be a critical factor in determining the DNA's effectiveness as a substrate for recombination (26,31). An ability of the topoisomerase to catalyze topological linking of complementary single-stranded rings of DNA also implies that this enzyme can act to resolve topological problems which might occur during recombination.

Breakage of single-stranded DNA to form a covalent protein-DNA complex is another reaction carried out by the Ustilago topoisomerase which may be biologically significant in recombinational processes. Type I topoisomerases from rat liver and HeLa cells form similar complexes

Figure 27. Topoisomerase activity in the rec 2 mutant.

Cells from haploid strains 58 (rec +) and 221 (rec 2) were grown in 2 liters of medium and processed through the polyethylene glycol step as described in Appendix D. Reactions (50 µl) containing 75 mM potassium phosphate, pH 7.5, 5 mM EDTA, 0.4 mM 2-mercaptoethanol, 50 µg/ml BSA, 5% glycerol, 2 nmol ϕχRF I DNA and the following amounts of enzyme were incubated at 25°C. AFter 30 min the reactions were terminated an analyzed by gel electrophoresis as described in Appendix C. Lanes A-C contained 1, 2, and 5 µg of protein from strain 58; lanes D-G contained 0.5, 1, 2, and 5 µg of protein from strain 221.


A B C D E F G

with single-stranded DNA and in addition they can covalently join the DNA in this complex to another DNA strand (14,39). Studies are in progress to determine if the Ustilago enzyme can also covalently join two DNA molecules.

Topoisomerase activity is significantly reduced in the recombination defective rec 2 mutant. Enzyme partially purified from this mutant is considerably less stable to heating than enzyme from rec + cells indicating that the mutant enzyme may be structurally altered.

The rec 2 mutant has a reduced frequency of mitotic crossing over. Although spontaneous gene conversion is normal, UV-induced gene conversion is substantially reduced. The most unusual feature of the rec 2 mutant is its inability to undergo meiosis. It has been speculated that the blockage in meiosis may be a result of an impaired ability of rec 2 to carry out rejoining of single strands of DNA at the late steps of chiasmata formation (92). The notion of topoisomerase playing a part in this step of meiotic recombination is tantalyzing.

APPENDICES

APPENDIX A
PHAGE, BACTERIAL AND FUNGAL STRAINS

Ustilago maydis 58 and 221 were acquired from Dr. Robin Holliday, National Institute for Medical Research, London. The cells were cultivated in medium consisting of 2% peptone, 2% sucrose, 1% yeast extract in a VirTis fermenter with vigorous aeration. Bacteriophage $\phi\chi$174am3 and its host E. coli HF4704 were acquired from Dr. Charles Radding, Yale University. Bacteriophage f∂ and its host E. coli K37 were acquired from Dr. W. Konigsberg. Bacteriophage P22 and its host Salmonella typhimurim DB25 were acquired from Dr. David Botstein, Massachusetts Institute of Technology.

APPENDIX B
PREPARATION OF DNA

Protocols used for preparing $\phi\chi$174am 3 phage DNA, f∂ phage DNA, 174 RFI [^{3}H]-DNA and f∂ RFI [^{3}H]-DNA were described by Cunningham et al. (93). Preparation of P22 [^{3}H]-DNA was described by Carter and Radding (94). Relaxed closed circular DNA (RF IV) was obtained by treating either $\phi\chi$ RF I or f∂ RF I DNA with purified Ustilago topoisomerase. Following relaxation, the topoisomerase was removed by phenol extraction. Conversion of f∂ RFI DNA to linear DNA(RFIII) was achieved by digestion with the restriction endonuclease Sau 96. Denaturation of duplex DNA was carried out by adding NaOH to 50 mM and incubating the DNA at 37°C. After 10 -15 min the DNA solution was reneutralized by adding HCl to 50 mM and Tris-HCl, pH 7.5 to 25 mM.

Labeling of $\phi\chi$ RF I DNA by nick translation with [α-^{32}P] dCTP used a modification of the procedure described by Muzyczka (95). Briefly, a reaction (55 µl) containing 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 25 µM dGTP, 25 µN dATP, 25 µM dTTP, 200 µCi [α-^{32}P] dCTP (410 ci/mmol), 3 nmol $\phi\chi$ RF I DNA, 1 ng pancreatic DNase, and 5 units E. coli DNA polymerase I were incubated at 15°C. After 1 hr, the reaction was terminated by adding EDTA to 15 mM. The reaction was then phenol extracted 1 time and the aqueous phase loaded onto a Sephacryl S-200 column equilibrated in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The DNA eluted in the void volume. The specific activity of the DNA was between 2.5 - 5.0 x 10^7 cpm/nmol DNA.

Labeling f∂ RF III at the 5' end was accomplished by using [γ-^{32}P] ATP and T4 polynucleotide kinase. Prior to kinasing, the DNA was dephosphorylated using bacterial alkaline phosphatase as described previously (96). Briefly, a reaction (80 µl) containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 8 nmol dephosphorylated f∂ RF III, 50 µCi [γ-^{32}P] ATP (3000 Ci/mmol), and 5 units of T4 polynucleotide kinase was incubated at 37°C for 90 min. The reaction was then dialyzed against 3 - 250 ml changes of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 M NaCl and then 3 - 250 ml changes of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The specific activity was 4×10^8 cpm/µmol.

Labeling f∂ RF III at the 3' end was accomplished by using [α-^{32}P] dGTP and the large Klenow fragment of E. coli DNA polymerase I (97). A reaction (80 µl) containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 µM dGTP, 125 µCi [α-^{32}P] dCTP (410 Ci/mmol) 25 nmol f∂ RF III DNA (Sau 96 restriction) and 3 units of the large Klenow fragment of E. coli DNA polymerase I was incubated at room temperature. After 10 min the reaction was stopped by adding 12.5 µl 0.2 M EDTA and 25 µl 20% sodium acetate, pH 6.0. The reaction volume was then adjusted to 150 µl and dialyzed as described previously for DNA labeled with T4 polynucleotide kinase. The specific activity of the DNA was 1×10^9 cpm/µmol.

Isolation of complementary single-stranded rings of ϕX DNA was achieved using a protocol described by Stettler et al. (98). Superhelical ϕX 174 DNA was nicked in one strand with pancreatic DNase in the presence of ethidium bromide. The strands were then separated by centrifugation through an alkaline sucrose gradient. The nicking

reaction (1 ml) contained 20 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 0.1 M NaCl, 300 μg/ml ethidium bromide, 800 nmoles $\phi\chi$RFI [^{3}H] DNA and 240 ng pancreatic DNAse. The reaction was incubated at 30°C for 20 min and then stopped by adding EDTA to 20 mM. The reaction was then phenol extracted 3 times to remove both protein and ethidium bromide. The DNA was precipitated from the aqueous phase by adding 2 volumes of ethanol and placing in a dry ice-ethanol bath for 10 min. After centrifugation, the DNA pellet was resuspended in 0.4 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Sodium hydroxide (10 M) was then added to a final concentration of 0.3 M. The denatured DNA was then divided in half and loaded on two 11-ml 5 - 20% sucrose gradients containing 0.25 M NaOH, 0.75 M NaCl, 1 mM EDTA. Gradients were centrifuged in the Ti40.1 rotor of a Beckman L5-50 centrifuge at 34,000 rpm for 18 hr at 4°C. Fractions (0.2 ml) were collected and the radioactive content of each was determined. The fractions containing the single-stranded circles were pooled and neutralized by adding 1 M Tris-HCl, pH 7.5.

APPENDIX C
ASSAYS

Topoisomerase

Topoisomerase activity was monitored during the enzyme purification using the D-loop assay. Reactions (50 µl) containing 75 mM potassium phosphate, pH 7.5, 1 mM EDTA, 5% glycerol and 3 nmol of ϕχRF I[^{3}H] DNA (1-2.5 x 10^4 cpm/nmol) were incubated with topoisomerase at 25°C. After 30 min, 10 µl of 3 M KCl was added to stop the reactions. The reaction was then split into two 30-µl aliquots. One aliquot was used to assess the amount of nuclease activity using an assay described by Kuhnlein et al. (45). To the second aliquot was added single-stranded fragments of ϕχ DNA to a final concentration of 0.2 mM. After incubating the reaction at 75°C for 3 hr the reaction mixture was diluted into 2 ml of cold 10 x SSC and passed through a nitrocellulose filter which had been previously washed with 5 ml 10 x SSC. Following two 5-ml washes with 10 x SSC the filters were dried and then counted in 2 ml of Econofluor. One unit of topoisomerase activity equals the amount of enzyme that prevents 1 nmol of ϕχ RF I[^{3}H] DNA from sticking to a filter. The efficiency of D-loop formation in the absence of added topoisomerase was approximately 50%.

The high salt nitrocellulose filter assay has also proven to be a convenient way to measure topoisomerase activity. After incubating the topoisomerase with ϕχ RF I [^{3}H]-DNA under the reaction conditions described in the D-loop assay, the reaction was split into two 25 µl aliquots. One aliquot was used to assess endonuclease activity as described before. The second aliquot was combined with 1 ml of 50 mM

Tris HCl, pH 7.5, 1 mM EDTA, 3.5 M NaCl and passed through a nitrocellulose filter previously washed with 5 ml of the same high salt buffer. After two 5-ml washes with the high salt buffer, the filter was dried and the radioactivity measured as in the D-loop assay.

The conversion of superhelical to relaxed DNA has also been followed using an agarose gel assay. Reactions (25 µl) were carried out using the same conditions as in the D-loop assay. To stop the reactions, 3 µl of a solution containing 5% SDS, 0.05% bromophenol blue, 50% glycerol was added. The sample was then electrophoresed through a 1.2% agarose gel containing 40 mM Tris-HCl, pH 7.9, 1 mM EDTA, 5 mM sodium acetate at 3V/cm for 12 - 14 hr at room temperature. Following electrophoresis the gel was stained with a 1 µg/ml solution of ethidium bromide for 10-30 min before photographing under ultraviolet light.

DNA-Binding Protein

The assay used to detect an interaction between protein and DNA has been described by Tsai and Green (99) and measures the retention of [^{3}H] DNA on a nitrocellulose filter in the presence of protein. To assay for DBP IV during the purification, reactions (50 µl) containing 20 mM Tris, pH 8.0, 1 mM EDTA, 2 mM 2-mercaptoethanol, 5% glycerol, 50 mM NaCl, and 2 - 3 nmol of denatured P22 [^{3}H] DNA (1.1×10^4 cpm/nmol) were incubated with DNA binding protein at 25°C. After 10 min the reactions were terminated by adding 1 ml of the reaction buffer and then processed as described by Banks and Spanos (100). One unit is defined as the amount of protein required to cause the retention of 1 nmol of DNA on a nitrocellulose filter.

Exonuclease

This assay measures the conversion of DNA to an acid-soluble form. Reactions with exonuclease I were carried out in 200 µl containing 50 mM glycine, pH 9.5, 10 mM $MgCl_2$ at 37°C. After 30 min the reactions were terminated by adding 0.3 ml of ice-cold carrier salmon sperm DNA (1 mg/ml) and 0.5 ml 10% trichloroacetic acid. After standing in ice for 10 min the tubes were centrifuged in an Eppendorf Microfuge. After 2 min, 0.5 ml of the supernatant was removed and counted in 5 ml of Triton scintillation fluor. Reactions with exonuclease VII were carried out in 200 µl containing 50 mM potassium phosphate, pH 7.9 at 37°C. After 30 min the reactions were terminated and processed as described for exonuclease I.

Protein

Protein content was measured using the method described by Bradford (101).

APPENDIX D
PROTEIN PURIFICATION

Topoisomerase

All of the steps during the purification were carried out between 0 - 4°C. Activity was determined using the D-loop assay. Cells (100 g) stored at -20°C were thawed in 200 ml of 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 M KCl, 5% glycerol (buffer A). The cells were broken in a French press at 20,000 psi and then centrifuged at 18,000 rpm for 30 min in the JA 20 rotor of a Beckman J21-C centrifuge (fraction I). After the lipid was removed from the supernatant, 24 g of polyethylene glycol was added and the mixture was allowed to stir for 4 hr before it was centrifuged. The supernatant was saved and the pellet was reextracted with 70 ml of buffer A and centrifuged again. The supernatants were combined (fraction II) and loaded onto a 130 ml hydroxylapatite column previously equilibrated in buffer A. The column was washed with 300 ml of buffer A before eluting the enzyme with a 1 liter gradient of (0.02 - 1.0 M) potassium phosphate, pH 7.0, in buffer A. The broad peak of activity (fraction III) which eluted about midway through the gradient was loaded directly onto a 40 ml octyl-Sepharose column equilibrated in 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, and 0.1 M KCl (buffer B). After washing the column with 200 ml of buffer B, a 200 ml 0-2% gradient of Triton X-100 was initiated. Activity eluted approximately two-thirds of the way through the gradient. The active fractions were pooled (fraction IV) and loaded directly onto a 2.5 ml phosphocellulose

column previously equilibrated in buffer B. After washing the column with 30 ml of buffer B, a 30 ml gradient from 0.1 - 1.0 M KCl in buffer B was applied. Activity eluted as a broad peak covering the latter third of the gradient. After pooling the active fractions (fraction V), a small aliquot (3 ml) was loaded onto a column of Sephacryl S-200 (1.4 x 56 cm) which had been previously equilibrated in buffer B containing 0.5 M KCl. Fractions (1 ml) were collected at a flow rate of 10 ml/hr. The peak of activity eluted between ferritin and catalase and was pooled (fraction VI).

25,000 Dalton Acid-Soluble Protein

The presence of the 25,000 dalton acid-soluble protein was determined at each step of the purification by SDS gel electrophoresis as described in Appendix E. All steps were carried out at 0 - 4°C. Cells (100 g) were suspended and broken in 300 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.35 M NaCl by passage through a French press at 20,000 psi and then centrifuged in the JA20 rotor of a Beckman J21-C centrifuge at 3500 rpm for 30 min. The supernatant was saved and made 5% in perchloric acid by adding 23 ml of 70% perchloric acid. After standing for 10 min the precipitate was removed by centrifugation. Solid trichloroacetic acid was then added to a final concentration of 25%. This was allowed to stir for 10 min before centrifuging. The pellet was saved and washed 1 time with 100 ml of acid acetone (1.0 ml concentrated HCl in 100 ml acetone) and 2 times with 100 ml of acetone. The residual acetone was removed under vacuum. The small white pellet was dissolved in 5 ml of 10 mM sodium borate, pH 9.0. Half of this volume was loaded onto a 2 ml carboxymethylcellulose column equilibrated in 10 mM sodium borate, pH

9.0, 0.1 M NaCl (buffer C). The column was washed with 20 ml of buffer C and then 10 ml of 0.2 M NaCl in buffer C. Finally the column was eluted with 10 ml of 0.5 M NaCl in buffer C and 1 ml fractions were collected. The 25,000 dalton protein eluted in fractions 2 and 3. These fractions were combined and contained approximately 60 μg of protein as judged from an SDS gel.

DBP IV

All steps of the purification were carried out at 0 - 4°C. The assay used to detect DBP IV is described in Appendix C. A summary of the purification is presented in Table VI. Frozen cells (100 g) were thawed and suspended in 200 ml of 20 mM potassium phosphate pH 7.0, 1 mM EDTA, 2 mM 2-mercaptoethanol, 0.1 mM PMSF, 5% glycerol (buffer D). The cells were broken in a Mantin-Gaulin cell crusher at 9000 psi and then centrifuged in the JA10 rotor of a Beckman J21-C centrifuge at 10,000 rpm for 30 min. The supernatant was discarded and the pellet resuspended in 100 ml of buffer D containing 1 M KCl and centrifuged again, this time in the JA 20 rotor at 18,000 rpm for 20 min. The pellet was discarded and to the supernatant was added solid polyethylene glycol to a final concentration of 7%. This mixture was allowed to stir for 2 hr and then centrifuged in the JA 20 rotor at 18,000 rpm for 20 min. The supernatant was saved and dialyzed against two 2-liter changes of 20 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM NaCl, 5% glycerol (buffer E). The dialysate (fraction I) was loaded onto a 15 ml DNA agarose column equilibrated in buffer E. After washing the column with 150 ml of buffer E containing 0.1 M NaCl, the column was eluted in

TABLE VI
PURIFICATION OF DBP IV

Fraction	Volume ml	Activity units	Protein mg	Specific Activity units/mg
I	100	2400	36.5	66
II	15	5100	0.5	10,200
III	10	9200	0.2[a]	46,000

[a]Protein concentration was estimated from an SDS polyacrylamide gel.

a stepwise fashion with 75 ml each of 0.25 M NaCl, 0.5 M NaCl, 1.0 M NaCl, and 2.0 M NaCl in buffer E. The eluate was collected in 5 ml fractions. DNA binding activity was present in both the 0.25 M and 0.5 M salt bumps. The active fractions from the 0.5 M salt bump were pooled and dialyzed against 1 liter of buffer E. The dialysate (fraction II) was then loaded onto a 5 ml carboxymethylcellulose column equilibrated in buffer E. After washing the column with 60 ml of buffer E, the column was washed in a stepwise manner with 35 ml of 0.25 M NaCl and then 1.0 M NaCl in buffer E. The eluate from the column was collected in 5 ml fractions. Fractions from the 1.0 M salt elution contained DBP IV and were pooled (fraction III).

APPENDIX E
ELECTROPHORESIS

Analysis of protein by SDS gel electrophoresis used the method described by Laemmli (102). Following electrophoresis the gels were usually fixed in ethanol/acetic acid/water (40/10/50, v/v/v) before staining with Coomassie blue. Occasionally, gels were stained with silver using the technique described by Merril et al. (103.)

Ionophoresis of acid hydrolysates on Whatman 3 MM paper was carried out at pH 3.5 in pyridine/acetic acid/H_2O (5/50/945, v/v/v) at 2000 V for 75 min (104). Following electrophoresis the paper was dried in a 50°C oven. Marker amino acids were visualized by spraying the paper with 0.3 % (wt/vol) ninhydrin in 1-butanol and then heating to 100°C for several minutes (105). Phosphate was detected by spraying the paper with a solution of 0.42% ammonium molybdate in 1 M sulfuric acid/10% ascorbic acid (6/1, v/v).

APPENDIX F
REAGENTS AND MATERIALS

Chromatographic Media

Affi-Gel blue, a Cibacron blue F3GA agarose was obtained from Bio-Rad Laboratories. Octyl-Sepharose and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals. Phosphocellulose P11 and carboxymethyl-cellulose were obained from Whatman. HA-Ultrogel was from LKB instruments.

Enzymes

Bacterial alkaline phosphatase and pancreatic DNase were obtained from Worthington Biochemicals Inc. E. coli DNA polymerase I was obtained from Boehringer Mannheim Biochemicals. Polynucleotide kinase, large Klenow fragment of E. coli DNA polymerase I, and restriction endonuclease Sau 96 were obtained from Bethesda Research Laboratories. Micrococcal nuclease and catalase were obtained from Sigma Chemical Co. Exonuclease I and Exonuclease VII were generously donated by M. Yarnell and S. Smallwood.

Miscellaneous

Histones, spermidine, poly-L-lysine, novobiocin, nalidixic acid, p-chloromercuriphenylsulfonate, 5,5'dithiobis(2-nitrobenzoate), ATP, GTP, UTP, TTP, dATP, ADP, AMP, dithiothreitol, N-ethyl maleimide phosphoserine and phosphothreonine were from Sigma Chemical Co. Electrophoresis grade agarose was from BioRad Laboratories. Poly(rA), poly(dG), poly(dC), poly(dA), poly(dT), β,γ-methylene adenosine triphosphate, α,β-methylene adenosine triphosphate and poly(d(G-C))

were obtained from P-L Biochemicals. Adenosine-5'-O-(3-thiotriphosphate), adenylylimidodiphosphate and CTP were from Boehringer Mannheim Biochemicals. Triton X-100 was from New England Nuclear. Nitrocellulose filters (BA85) were from Schleicher and Schuell. Phosphothreonine was kindly provided by Dr. D. Rawlins. Berenil was a generous gift from the Squibb Pharmaceutical Co. Phosphotyrosine was synthesized according to Rothberg et al. (105).

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BIOGRAPHICAL SKETCH

Tom was born on December 8, 1951, the fourth child of John Rowe and Marie Cardon. He has three older brothers, John, Rich and Bob, as well as a younger sister Beverly. Tom was raised in Boulder, Colorado, and graduated from Boulder High School in 1970. He then attended Colorado State University where he received his Bachelor of Science degree in chemistry. Since then, Tom has been a graduate student working in the laboratory of Dr. Bill Holloman in the Department of Immunology and Medical Microbiology at the University of Florida. Upon receiving his degree of Doctor of Philosophy, Tom will join the laboratory of Dr. Leroy Liu in the Department of Physiological Chemistry at The Johns Hopkins University School of Medicine in Baltimore, Maryland.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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December 1982

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